January, 1950 ~~Price 20s. net~~ Vol. 4, No. 1

THE JOURNAL OF GENERAL MICROBIOLOGY

QR
1
J64

EDITED FOR

THE SOCIETY FOR GENERAL MICROBIOLOGY

Editors

B. C. J. G. KNIGHT A. A. MILES

Associate Editors

G. C. AINSWORTH
W. B. BRIERLEY
T. GIBSON
A. T. R. MATTICK
K. M. SMITH
A. W. STABLEFORTH
A. F. B. STANDFAST
D. D. WOODS

CAMBRIDGE UNIVERSITY PRESS
(CAMBRIDGE, AND BENTLEY HOUSE, LONDON)
CANADA AND INDIA: MACMILLAN

THE
JOURNAL OF GENERAL MICROBIOLOGY

is conducted by the

SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology was founded in order to promote the advancement of microbiology by providing a common meeting ground for those working in all the various specialized fields including, for example, medical, veterinary, agricultural and economic microbiology. The Society is concerned with the more fundamental aspects of the study of bacteria, viruses, micro-fungi, protozoa and microscopic algae in their various biological activities, its members being united by the common aim of developing scientific knowledge concerning micro-organisms.

The annual subscription, which entitles members to receive the *Journal of General Microbiology*, is 35*s*. and is payable on 25th March each year.

OFFICERS OF THE SOCIETY

President

J. W. McLeod, F.R.S.

Hon. Treasurer

H. J. Bunker, M.A., Barclay Perkins Ltd., Park St., London, S.E. 1

Hon. Secretaries

J. G. Davis, D.Sc., 35 Villiers Road, Southall, Middlesex

W. E. van Heyningen, Ph.D., Sir William Dunn School of Pathology, Oxford University.

All requests for general information should be sent to J. G. Davis and all communications concerning meetings of the Society should be sent to W. E. van Heyningen.

PURCHASE OF JOURNAL BY NON-MEMBERS

For non-members of the Society the subscription for the *Journal of General Microbiology* is £2. 10*s*. 0*d*. per volume. Single parts may be purchased for 20*s*. plus postage.

Enquiries about subscriptions should be addressed to the

CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE
200 EUSTON ROAD, LONDON, N.W. 1

or through any bookseller.

Enquiries from the U.S.A. should be addressed to the Cambridge University Press, American Branch, 51 Madison Avenue, New York 10.

Applications for advertising space should be sent to the Cambridge University Press.

THE JOURNAL OF GENERAL MICROBIOLOGY

EDITED FOR

THE SOCIETY FOR GENERAL MICROBIOLOGY

Editors

B. C. J. G. KNIGHT A. A. MILES

Associate Editors

G. C. AINSWORTH
W. B. BRIERLEY
T. GIBSON
A. T. R. MATTICK
K. M. SMITH
A. W. STABLEFORTH
A. F. B. STANDFAST
D. D. WOODS

VOLUME 4, 1950

CAMBRIDGE
AT THE UNIVERSITY PRESS
1950

Printed in Great Britain at the University Press, Cambridge
(Brooke Crutchley, University Printer)
and published by the Cambridge University Press
London: Bentley House, N.W. 1
American Branch: New York
Agents for Canada, India and Pakistan: Macmillan

Kernforschungsanlage
Jülich
Zentralbibliothek

Pr 2770/ZB (PC/Prof. Haul)

Contents

No. 1 (*issued* 24 *January* 1950)

PAGE

The Sporulation of *Clostridium tetani*. By K. A. Bisset. (With one plate) 1

An Investigation of Pleuropneumonia-like Organisms Isolated from the Bovine Genital Tract. By D. G. ff. Edward. (With one plate) . 4

The Influence of the Bacterial Environment on the Excystment of Amoebae from Soil. By Lettice M. Crump 16

The Relation of Toxicity and Enzyme Activity in the Lecithinases of *Clostridium bifermentans* and *Clostridium welchii*. By Ellen M. Miles and A. A. Miles. (With three figures) 22

A Note on the Globular Forms of *Vibrio cholerae*. By the late P. Bruce White. (With one plate) 36

Chemical Factors in the Germination of Spore-bearing Aerobes: Observations on the Influence of Species, Strain and Conditions of Growth. By G. M. Hills. (With one figure) 38

The Non-toxicity of *Bacillus anthracis* Cell Material. By H. K. King and Jean H. Stein 48

Interpreting Relationships between the Concentrations of Plant Viruses and Numbers of Local Lesions. By A. Kleczkowski. (With five figures) 53

The Assay of the Antibiotic Nisin. By A. Hirsch. (With five figures) 70

Some Aspects of Cell Division in *Saccharomyces cerevisiae*. By A. A. Barton. (With one plate) 84

The Reducing Sugars Liberated during the Bacterial Synthesis of Polysaccharides from Sucrose. By W. G. C. Forsyth and D. M. Webley 87

Classification of the Streptococci of Subacute Bacterial Endocarditis. By J. S. Porterfield 92

The Effect of the Gas-Phase on Differential Inhibition of Intestinal Bacilli. By J. Brodie and W. Shepherd 102

Proceedings of the Society for General Microbiology.
Nottingham, 29 *and* 30 *September* 1949

Original Papers

Studies on the Correlation of the Physical and Biological Properties of *Haemophilus pertussis*. By J. Ungar, A. M. James and W. K. Stevens i

Aspects of the Trace-element Nutrition of *Streptomyces griseus*. By C. G. C. Chesters and G. N. Rolinson i

PAGE

Nutrition of *Clostridium tetanomorphum*. By K. A. Sims and D. D. Woods ii

The Release of Cellular Constituents from *Staphylococcus aureus* treated with Cetyltrimethylammonium Bromide. By M. R. J. Salton and A. E. Alexander ii

The Bacterial Surface: Effect of Cetyltrimethylammonium Bromide on the Electrophoretic mobility. By K. McQuillen iv

The Formation of Ethyl Acetate by Yeast. By J. L. Peel . . iv

The Assimilation of Glutamic Acid Derivatives by *Staphylococcus aureus*. By E. F. Gale v

Further Observations on the Group or Species Specific Ninhydrin-Positive Substances produced by Bacteria. By A. J. Woiwod and H. Proom v

Do *Fusiformis necrophorus* and *Streptobacillus moniliformis* show a Primitive Form of Sexuality according to Mellon's views? By E. Klieneberger-Nobel vi

Pure Culture Isolation of *Nitrosomonas*. By Jane Meiklejohn . . vi

A Tentative Method of obtaining a 'Balance Sheet' of the Soil Microflora. By C. G. C. Chesters and A. Apinis vi

Measurements of Rate of Mutation of Flagellar Antigenic Phase in *Salmonella typhi-murium*. By B. A. D. Stocker vii

Some Observations on the Classification of the Genus *Bacillus*. By H. Proom viii

The Mechanical Transmission and some properties of Potato Paracrinkle virus. By F. C. Bawden, B. Kassanis and H. L. Nixon . . viii

Observations on the Cultivation of *Haemophilus pertussis*. By Betty Dawson, E. Farnworth, D. E. Nicholson and J. W. McLeod . viii

Non-specific Lysis observed in the Bacteriophage typing of *Staphylococcus aureus*. By Joan E. Rippon viii

Osmophilic Yeasts from Concentrated Orange Juice. By M. Ingram . ix

Demonstrations ix

No. 2 (*issued* 11 *May* 1950)

Obituary Notice—S. Orla-Jensen, 1870–1949. By E. Olsen. (With one plate) 107

The Bacteriostatic Action of Phenol, Benzoic Acid and Related Compounds on *Bacterium aerogenes*. By R. G. H. Barbour and J. M. Vincent. (With five figures) 110

PAGE

The Antibiotic Properties of Fifty-two Strains of *Fusarium*. By MARGARET S. LACEY 122

Factors Affecting the Fruiting of *Chaetomium* Species. By S. N. BASU and R. G. BOSE 132

Some Observations on a Streptomycin-dependent Strain of *Staphylococcus aureus*. By G. E. FOLEY and H. SHWACHMAN. (With one plate) 141

Studies of a Yeast Exacting towards *p*-Aminobenzoic Acid. By N. S. CUTTS and C. RAINBOW 150

The Degradation of Starch by Strains of Group A Streptococci having Related Antigens. By NUALA CROWLEY 156

The Effect of Growth-factor Deficiencies upon Fermentation of Glucose by Yeasts. By R. H. HOPKINS and R. J. PENNINGTON. (With twelve figures) 171

The Isolation of *Nitrosomonas europaea* in Pure Culture. By JANE MEIKLEJOHN. (With one plate) 185

Acetobacter acidum-mucosum Tosic & Walker, n.sp., an Organism Forming a Starch-like Polysaccharide. By J. TOSIC and T. K. WALKER. (With one plate) 192

The Bacterial Genus *Lineola*. By E. G. PRINGSHEIM. (With ten figures) 198

The Mechanical Transmission and some Properties of Potato Paracrinkle Virus. By F. C. BAWDEN, B. KASSANIS and H. L. NIXON. (With one plate) 210

An Electron-Microscope Study of Potato Virus *X* in Different States of Aggregation. By A. KLECZKOWSKI and H. L. NIXON. (With two plates) 220

Further Observations on a Filtrable Agent Isolated from Bovine Lumpy Skin Disease. By M. VAN DEN ENDE and G. S. TURNER. (With one figure) 225

An Improved Method for the Preparation of Silica Gel Media for Microbiological Purposes. By C. B. TAYLOR. (With one plate) . . 235

The Production of γ-Aminobutyric Acid by *Bacterium coli* Wilson, Type I. By H. K. KING and L. I. FLETCHER 238

A Note on Stalked Forms of Viruses. By C. F. ROBINOW. (With one plate) 242

The Distinction of Licheniformin from Subtilin by Cross-Reactions with Antibiotic-Resistant Strains of *Mycobacterium phlei*. By P. D'ARCY HART and BRENDA MOSS. (With two figures) 244

The Morphology and Motility of *Proteus vulgaris* and Other Organisms Cultured in the Presence of Penicillin. By A. FLEMING, AMALIA VOUREKA, I. R. H. KRAMER and W. H. HUGHES. (With three plates and four figures) 257

PAGE

A Growth-Inhibitory Effect on *Shigella dysenteriae* which Occurs with some Batches of Nutrient Agar and is Associated with the Production of Peroxide. By H. Proom, A. J. Woiwod, Joan M. Barnes and W. G. Orbell 270

The Utilization of Amino-Acid Solutions by Virus-Infected Eggs, Studied by Paper Chromatography. By M. van den Ende . . . 277

Jensenia, A New Genus of the Actinomycetales. By K. A. Bisset and F. W. Moore 280

No. 3 (*issued* 30 *September* 1950)

A New Aquatic Nitrogen-Fixing Bacterium from Three Cambridgeshire Chalk Streams. By E. Gray and J. D. Smith. (With one plate) . 281

Anaerobic and Aerobic Growth of Purple Bacteria (Athiorhodaceae) in Chemically Defined Media. By S. H. Hutner 286

The Serological Identity of a Yellow-Pigmented *Streptococcus*. By C. L. Hannay 294

A Study of Phage-Resistant Mutants of *Rhizobium trifolii*. By J. Kleczkowska. (With one figure) 298

An Investigation of the Biological Properties of Organisms of the Pleuropneumonia Group, with Suggestions Regarding the Identification of Strains. By D. G. ff. Edward 311

Factors Affecting the Germination of Thick Suspensions of *Bacillus subtilis* Spores in L-Alanine Solution. By Joan F. Powell. (With one plate and four figures) 330

Colony Counts on Strips of Agar in Tubes. By Anna C. Stirling, Marion K. Stevens and D. N. Lawley 339

The Cultivation of *Haemophilus pertussis* in Partially-defined Liquid Media. By J. Ungar, A. M. James, P. W. Muggleton, H. F. Pegler and E. G. Tomich. (With one plate and six figures) . 345

Enzymes of *Clostridium welchii* Type A and *Clostridium histolyticum* that Disintegrate Decalcified Human Tooth Dentine. By D. G. Evans and A. S. Prophet 360

The Alpha, Beta and Gamma Antigens of *Clostriduim histolyticum* (Weinberg & Séguin, 1916). By C. L. Oakley and G. Harriet Warrack 365

Some Factors Influencing the Rate of Formation of Tetrathionase. By W. H. H. Jebb, R. Knox and A. H. Tomlinson. (With ten figures) 374

Tetrathionase: The Differential Effect of Temperature on Growth and Adaptation. By R. Knox. (With two figures) 388

PAGE

Simplified Fluorescence Microscopy of Tubercle Bacilli. By E. MATTHAEI. (With one plate) 393

Spectrophotometric Estimation of Nucleic Acid in Bacterial Suspensions. By P. MITCHELL. (With seven figures) 399

A Versatile Fermentation Sampling Arrangement. By N. G. HEATLEY. (With one figure) 410

The Differentiation of Certain Genera of Bacteriaceae by the Morphology of the Microcyst Stage. By K. A. BISSET. (With one plate and one figure) 413

Marcescin, an Antibiotic Substance from *Serratia marcescens*. By A. T. FULLER and JEAN M. HORTON 417

Red-Leg in Tree-Frogs Caused by *Bacterium alkaligenes*. By ELLEN M. MILES 434

Factors Influencing the Early Phases of Growth of *Aerobacter aerogenes*. By S. DAGLEY, E. A. DAWES and G. A. MORRISON. (With five figures) 437

An Improved Colony Illuminator. By W. T. MOORE and C. B. TAYLOR. (With one figure) 448

Viability of Dried Bacterial Cultures. By MABEL RHODES. With a Note on the Immediate Death-Rate. By P. J. FISHER. (With one figure) 450

Further Observations on the Motility of *Proteus vulgaris* Grown on Penicillin Agar. By A. FLEMING. (With three figures) 457

Some Factors Affecting the Activation of Virus Preparations Made from Tobacco Leaves Infected with a Tobacco Necrosis Virus. By F. C. BAWDEN and N. W. PIRIE 464

Some Effects of Freezing in the Leaf, and of Citrate *in vitro*, on the Infectivity of a Tobacco Necrosis Virus. By F. C. BAWDEN and N. W. PIRIE. (With one figure) 482

A Preliminary Study of Ammonia Production by *Corynebacterium renale* and some other Pathogenic Bacteria. By R. LOVELL and D. G. HARVEY. (With five figures) 493

Identification of Characteristic Extracellular Ninhydrin-Positive Substances Produced by some Bacteria. By A. J. WOIWOD and H. PROOM. (With two plates) 501

A Comparative Survey of the Nutrition and Physiology of Mesophilic Species in the Genus *Bacillus*. By B. C. J. G. KNIGHT and H. PROOM 508

Bacillus pantothenticus (n.sp.). By H. PROOM and B. C. J. G. KNIGHT. (With one plate) 539

PAGE

Proceedings of the Society for General Microbiology. London, 4 and 5 April 1950

ORIGINAL PAPERS

The Decarboxylation of Succinic Acid by Washed Suspensions of Rumen Bacteria. By S. R. ELSDEN and A. KAARS SYPESTEYN . xi

The Reduction of Nitrate by Rumen Bacteria. By DYFED LEWIS . xi

*A Possible New Species in the Genus *Bacillus*. By H. PROOM and B. C. J. G. KNIGHT xii

Further Comparative Study of Nutritional Requirements in the Genus *Bacillus*. By B. C. J. G. KNIGHT and H. PROOM xii

Resistance to Infection with *Salmonella enteritidis* and *Mycobacterium tuberculosis* of Mice Fed Different Diets. By J. W. HOWIE . . xii

The Production of Non-Capsulated Avirulent Variants by *Bacillus anthracis* and its Implication on Taxonomy. By H. P. CHU . . xiii

The Deamidation of Nicotinamide by *Lactobacillus arabinosus* 17–5. By D. E. HUGHES and D. H. WILLIAMSON xiv

The Formation and Germination of Bacterial Spores Studied by Phase Contrast and Slide-Cell Culture. By R. J. V. PULVERTAFT . . xiv

Cytological Studies on Tissue Cultures Infected with Fowl-Plague Virus. By C. E. CHALLICE and T. H. FLEWETT xiv

The Serology of *Bacillus polymyxa*. By SHEILA N. DAVIES . . . xv

The Measurement of the Aeration of Biological Culture Media. By W. S. WISE xv

The Two Opposing Effects of Subtilin on Bacteria. By T. M. ASHER xvi

*The Control of Pyrogen Formation During the Refining of Antitoxic Sera. By A. E. FRANCIS xvi

Bacterial Macromolecules. I. The Isolation of Deoxyribonucleic Acid from Virulent and Avirulent Strains of *Haemophilus pertussis*. By W. G. OVEREND, M. STACEY, M. WEBB and J. UNGAR . . xvi

Induced Mutation of *Bacillus anthracis*. By J. TOMCSIK . . . xvi

Some Effects of Cobalt on the Growth and Metabolism of *Proteus vulgaris*. By A. L. SCHADE and H. B. LEVY xvii

FILMS AND DEMONSTRATIONS xviii

No. 3, Volume 3 of the Journal of General Microbiology was issued on 27 September 1949

The Sporulation of *Clostridium tetani*

By K. A. BISSET
Department of Bacteriology, University of Birmingham

SUMMARY: In the sporulation of *Clostridium tetani*, a rod-like fusion nucleus is formed from the two nuclear units typical of a 'smooth' bacillus. The fusion nucleus divides into two smaller rods, one of which degenerates; the other is included in the spore. The nucleus remains rod-shaped until the maturation of the spore is almost complete, when it is transformed into a spherical, eccentric spore nucleus.

The cytology of sporulation in certain types of bacillus is well known (Badian, 1933; Klieneberger-Nobel, 1945; Flewett, 1948), and it is generally agreed that the four nuclear units of the bacillus form a rod-shaped fusion nucleus, from which, usually after a cell division, the four units are regenerated. One unit is enclosed in the spore and the other three are rejected.

In my experience this scheme is applicable only to bacilli of 'rough' morphology, which are multicellular and have one nuclear unit in each cell, and not to those of 'smooth' morphology, which possess two nuclear units in each unicellular bacillus (Bisset, 1948).

The present study describes the sporulation of *Clostridium tetani*, a species which is normally 'smooth', and which also differs from the species previously described in producing a very large terminal spore. The large size of the spore enabled the behaviour of its nuclear material, during maturation, to be observed with reasonable clarity.

MATERIALS AND METHODS

Two strains of *Cl. tetani* were used. One was isolated from a fatal case of tetanus in man, the other from horse-dung. They were grown anaerobically upon pepsinized blood-agar, at 37°. Preparations were stained by the acid-Giemsa technique.

Observations

Sporulation was well advanced in cultures 24 hr. old (Pl. 1, figs. 1, 2). Vegetative cells contained the two pairs of chromosomes typical of the smooth morphology (Fig. 1*a*). The formation of the fusion nucleus (Fig. 1*c*) was preceded by a stage in which an irregular, broken rod was formed (Fig. 1*b*). The fusion nucleus then assumed its compact rod shape, and divided into two (Fig. 1*d*). Up to this point the process was exactly comparable with that described by Klieneberger-Nobel; but thereafter was markedly different. The division of the nuclear rod was not followed by division of the bacillus, which remained entire. One of the daughter rods retained its form, and became the nucleus of the spore, the other became spherical in form, and then disintegrated (Pl. 1, fig. 3; and Fig. 1*e*, *f*). The granular remains of this disintegrated nuclear unit were often seen in bacilli containing young spores (Pl. 1, fig. 2).

The remaining nuclear unit retained its rod shape, and was surrounded by a sharply bounded. refractive area which increased in size until it completely filled the swollen extremity of the bacillus (Pl. 1, fig. 2; and Fig. 1*f*, *g*, *h*). As the spore matured, the nuclear body was transformed from a rod to an eccentric, spherical body (Pl. 1, figs. 2, 3; and Fig. 1*h*, *i*, *j*).

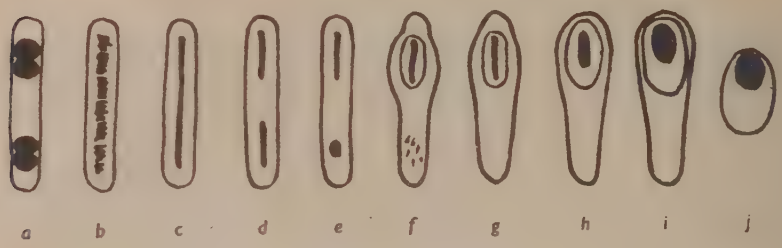


Fig. 1. Sporulation in *Cl. tetani*.

DISCUSSION

In the sporulation of *Cl. tetani* it appears that the formation of a rod-shaped fusion nucleus, a short-lived diploid stage, is followed by a reduction division, in which half the nuclear material is rejected. This process differs from that described by several authors as occurring in bacilli of 'rough' morphology, in which a cell division may precede sporulation, and in which three out of four nuclear units are rejected. It differs also in that the discarded nuclear material is absorbed into the cytoplasm, whereas in the 'rough' forms of other species its disappearance accompanies the dissolution of the bacillus and release of the spore.

The behaviour of the spore nucleus during maturation is of interest. The retention of the rod shape within the almost spherical spore may be taken to indicate that this nuclear form is not simply that imposed upon a mass of nuclear material lying within a rod-shaped cell, but that it possesses some structural coherence. The drumstick appearance of the sporing bacillus, which is not seen in water-mounted preparations, is presumably an artefact due to drying.

REFERENCES

Badian, J. (1933). Eine cytologische Untersuchung über das Chromatin und den Entwicklungszyklus der Bakterien. *Arch. Mikrobiol.* **4**, 409.

Bisset, K. A. (1948). The cytology of smooth and rough variation in bacteria. *J. gen. Microbiol.* **2**, 83.

Flewett, T. H. (1948). Nuclear changes in *Bacillus anthracis* and their relation to variants. *J. gen. Microbiol.* **2**, 325.

Klieneberger-Nobel, E. (1945). Changes in the nuclear structure of bacteria, particularly during spore formation. *J. Hyg., Camb.*, **44**, 99.

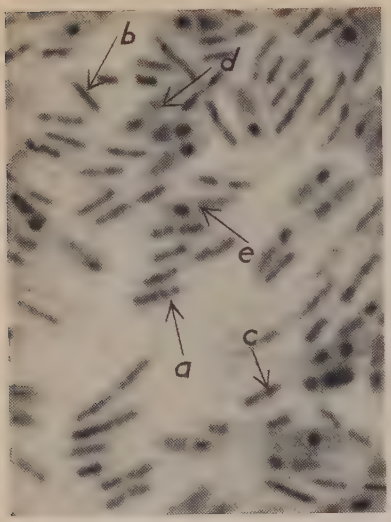


Fig. 1

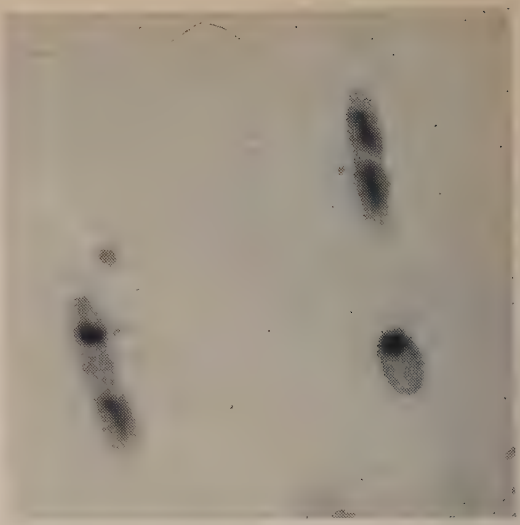


Fig. 3

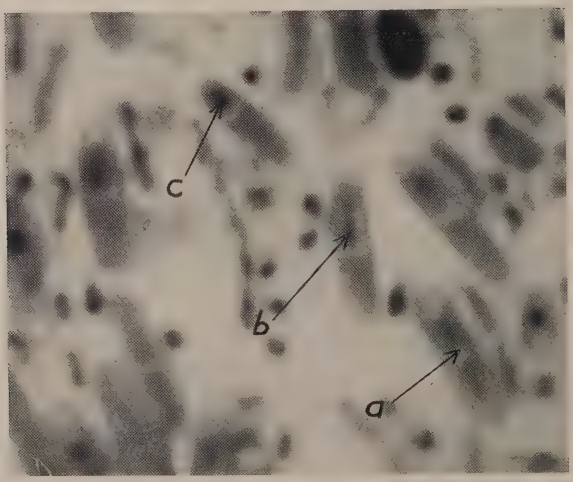


Fig. 2

K. A. Bisset—The sporulation of *Cl. tetani*. Plate 1

EXPLANATION OF PLATE

Fig. 1. *Cl. tetani*, vegetative cells and stages in sporulation. ×3000. (*a*) Vegetative cell; (*b*) fusion nucleus; (*c*) fusion nucleus after division; (*d*) early stage of spore formation; (*e*) later stage of spore formation.

Fig. 2. *Cl. tetani*, stages in sporulation. ×6000. (*a*) Enclosure of one nucleoid in spore, degeneration of the other; (*b*) early spore containing rod-shaped nucleus; (*c*) later spore, containing spherical nucleus.

Fig. 3. *Cl. tetani*, one cell showing divided fusion nucleus, the other showing half of the nucleus commencing to degenerate. Also a mature spore. ×6000.

(*Received* 18 *February* 1949)

An Investigation of Pleuropneumonia-like Organisms Isolated from the Bovine Genital Tract

By D. G. ff. EDWARD
The Wellcome Veterinary Research Station, Frant, Sussex

SUMMARY: Two members of the pleuropneumonia group, provisionally called the P and S species, were isolated from the genital tract of cattle.

The P strains required serum for artificial cultivation. Cultures on horse serum media had a characteristic appearance, due to the precipitation of a substance, probably protein; this aided identification. Strains differed antigenically, although common antigens were sometimes shared; antisera were prepared in rabbits against three different serological types. Agglutinins for P strains were not found in sera of infected cattle. There is some evidence that these strains may be capable of causing an inflammation of the genital tract which predisposes to infertility.

The S strains resembled the saprophytic members of the pleuropneumonia group, to which they were serologically related; they grew at room temperature and on media devoid of serum. They were antigenically distinct from P strains. They may be commensals or gain access to discharges as contaminants.

The observations in cattle suggest further study of strains of pleuropneumonia-like organisms isolated from the human genital tract. The assessment of their pathogenicity for man may be complicated by the presence of more than one species in the human genital tract.

Since the finding of pleuropneumonia-like organisms in the genital tract of cattle was first reported (Edward, Hancock & Hignett, 1947) investigations have been made to assess their significance and to determine whether they may be a cause of infection of the genital tract and consequently of infertility. In the course of these investigations the isolation and maintenance in subculture of a number of strains of pleuropneumonia-like organisms have provided information concerning their properties. It was apparent that there were at least two species among strains isolated from the genital tract of cattle. They differed in a number of respects, the most striking difference being that, whereas one type needed for growth in artificial culture a medium enriched by serum or ascitic fluid, the other grew well on media devoid of serum. All members of the pleuropneumonia group, previously isolated from animals or from cultures of other bacteria and including the known pathogens, have needed serum for growth in artificial culture. Only the 'saprophytic' strains, isolated by Laidlaw & Elford (1936) from sewage and by Seiffert (1937*a*, *b*) from soil and decaying vegetable matter, could be grown on a serum-free medium. This paper records observations, including the results of a serological investigation, made on a number of strains isolated from the bovine genital tract.

TECHNICAL METHODS

The methods used for the isolation of pleuropneumonia-like organisms from the genital tract of cattle have already been described in detail (Edward *et al.* 1947). The semen of bulls and the vaginal mucus of cows were cultured on selective

media. Isolations were made from single colonies; as a routine, strains were subcultured at weekly intervals on solid media, the plates being incubated for 2–3 days and then stored at 4° for the remainder of the week. As a precaution against loss each strain was also maintained by weekly subculture in a semi-solid medium (0·3 % agar).

The media employed were described by Edward (1947). To a basal medium prepared from ox-heart infusion broth with the addition of 1 % peptone, 10 % yeast extract and 20 % (v/v) horse serum were added, the reaction being finally adjusted to pH 8·0. Penicillin (2 drops of a solution containing 1000 u./ml. spread over half a plate of solid medium and 100 u./ml. added to a semi-solid medium) and thallium acetate (1/8000 in solid media and 1/2000 in semi-solid and fluid media) were used to inhibit the growth of other bacteria. For special purposes human ascitic fluid (20 %, v/v) and rabbit serum (10 %, v/v) replaced horse serum.

Dienes (1947 *a*, *b*) claimed that under the action of penicillin pleuropneumonia-like organisms could develop from cultures of other bacteria, particularly *Haemophilus influenzae*. In the present investigation there was no evidence that the strains isolated originated from the use of penicillin in the selective medium. For primary isolation a specimen was spread over the whole of a plate, only half of which had been previously treated with penicillin. Colonies of pleuropneumonia-like organisms appeared on the untreated half as well as on the treated side, except where the untreated half was overgrown by other bacteria.

Preparation of antigens. Cultures were made in the special broth, enriched with 20 % (v/v) human ascitic fluid and containing 1/2000 thallium acetate; approximately 200 ml. of medium was inoculated with a piece of agar from a young plate culture on an ascitic fluid medium. After incubation for a number of days until growth was maximal, the cultures were centrifuged (angle centrifuge, 60 min. at 4000 r.p.m.) and the deposit was washed once by suspending in saline and recentrifuging. For agglutination the deposit was finally suspended in buffered formol saline (Klieneberger, 1938), the suspension having an opacity half that of a Brown's tube no. 1 (Burroughs, Wellcome and Co.). Uniform and relatively stable suspensions were readily obtained; occasionally low-speed centrifugation for a few minutes was necessary to get rid of a few large clumps.

For complement-fixation tests the washed deposit of organisms was suspended in normal saline containing 1/1000 merthiolate, the dilution in the first place being such that the suspension had an opacity equal to Brown's tube no. 2.

Preparation of rabbit antisera. To avoid inducing in the rabbit antibodies to foreign serum protein, all cultures used for immunization were grown in broth enriched with rabbit serum only. Unfortunately, most strains, after isolation and maintenance on media containing horse serum, at first grew poorly, or not at all, on media made with rabbit serum; gradual adaptation was necessary. A strain was first grown on a medium containing 10 % (v/v) rabbit serum and 2 % (v/v) horse serum. When growth was established the proportion of horse

serum in the medium was diminished until it could be omitted. It was only after prolonged subculture, 2 or 3 times a week for weeks or months, that adequate growth was obtained in 10 % (v/v) rabbit serum broth.

The growth, after centrifugation and resuspension in saline, was injected intravenously. Between 5 and 10 doses, using the deposits from a total of 0·5–1·0 l. of culture, were necessary before sufficiently high titres of antibodies developed. It was usual to inject a rabbit on each of two successive days and then to wait 7 to 10 days before giving another two injections. The health of the rabbits was unaffected by the inoculations of the living cultures.

Agglutination test. The tests were made in Dreyer's tubes, containing 0·1 ml. of successive two-fold dilutions of serum and 0·1 ml. of suspension. Agglutination was read after 4 and 6 hr. in the water bath at 52–56°. It was easily seen by the naked eye when the tubes were examined in a dark room with a bright light. The end-point of the titration was confirmed using a hand lens. This technique proved to give the best results after other methods had been tried. Incubation at 37° in the water bath was effective for some antigens, but others were not completely agglutinated at this temperature. Allowing the tubes to stand at room temperature overnight before the final reading (Klieneberger, 1938) resulted in a non-specific clumping on the sides of the tubes which made reading difficult. Suspensions differed in the case with which they were agglutinated and in the type of clumping. Well-marked prozones up to a dilution of 1/64 sometimes occurred with high-titre sera. In all the tests controls with saline and normal rabbit serum were included.

Complement-fixation test. The tests were made in $2\frac{3}{4} \times \frac{3}{8}$ in. tubes using unit volumes of 0·125 ml. In the test proper 1 vol. of antigen and 2 vol. of complement were added to 1 vol. of serum dilution. After incubation for 2 hr. at 37°, 2 vol. of sensitized sheep red cell suspension (3 % suspension of washed cells mixed 30 min. previously with an equal volume of saline containing 12 M.H.D./ml. of haemolysin) were added and the tests read after further incubation for 1 hr. Guinea-pig serum preserved by Richardson's method (1941) was the source of complement. It was titrated with saline and with each antigen separately using the same volumes as in the test proper. Little difficulty was experienced with anti-complementary antigens. For the tests $1\frac{1}{2}$ doses of complement were used, 1 dose being the smallest amount giving complete haemolysis in the preliminary titration with that particular antigen. In the performance of every test and titration adequate control tubes were included. Rabbit and bovine sera were inactivated by heating to 62° for 30 min.

The optimum dilution for each antigen was estimated, using its homologous serum or some other rabbit antiserum with which it fixed complement. A series of titrations of serum were put up using different dilutions of antigen, namely, 1/1, 1/2, 1/4 and 1/8. It was found that whereas some antigens needed no dilution, others gave higher serum titres when diluted 1/2 or 1/4; one antigen fixed complement completely only after it had been concentrated twofold by centrifugation. In the tests each antigen was used at its optimum dilution.

RESULTS

Cultural characters of P and S strains

Many strains of pleuropneumonia-like organisms were isolated from a large number of cattle, but technical difficulties made it impossible to maintain and investigate more than a selected few. It was soon noted that the majority produced peculiar and characteristic effects when grown on horse serum media; others lacked this property. The two types of organism also differed in other respects, notably in nutritional requirements. Whereas the strains producing the peculiar changes in horse serum media needed serum for growth, the other strains grew well without it. For convenience the former are referred to as 'P' strains and the latter as 'S' strains.

The P strains grew satisfactorily with horse serum or human ascitic fluid, but irregularly with ox serum. They grew on rabbit serum media, only after gradual adaptation; strains varied in the ease with which they could be adapted.

The S strains grew well on solid media without serum, although serum was necessary for adequate growth in fluid media. They resembled the saprophytic members of the pleuropneumonia group in this respect and in their ability to grow at room temperature; no growth of P strains occurred at this temperature. When grown in the presence of horse serum the S strains produced none of the peculiar changes in the medium (see below) always produced by P strains.

P and S strains produced haemolysis when grown as deep colonies in the special horse serum medium containing 5 % (v/v) defibrinated blood. Saprophytic sewage organisms produced similar haemolysis. Haemolysis was obtained with human, horse, ox, sheep and rabbit blood. Haemolytic colonies of pleuropneumonia-like organisms were seen in pour-plates of ordinary blood agar, used to isolate and diagnose *Corynebacterium pyogenes* in vaginal discharges. The colonies could be easily distinguished under a magnification of $\times 10$; colonies of pleuropneumonia-like organisms were small, translucent and circular, and those of *C. pyogenes* opaque and irregular.

Changes in horse serum media produced by P strains

All P strains produced characteristic effects when grown on horse serum media. On solid media, after *c.* 4 days' incubation a greyish white film appeared over the surface of the heavily inoculated portion of an agar-plate culture; this could easily be seen by the naked eye (Pl. 1, fig. 1). When examined with the dissecting microscope (magnification $\times 10$) this film appeared crinkled and extended between the colonies of the organism; there were also numerous small black dots in the upper layer of the medium both beneath the colonies and a few millimetres away, suggesting that they had been caused by a substance diffusing from the colony (Pl. 1, fig. 2). Fluid cultures developed an opalescence denser than is usual from the growth of a pleuropneumonia-like organism. Dark-ground microscopy revealed numerous relatively highly refractile masses in addition to the typical pleomorphic elements of the organism. They were mostly *c.* 4 μ in diameter; sometimes they were smaller and more loosely constructed, when they appeared to consist of separate elements, whose shape it

was difficult to determine. One or more spherical masses were sometimes seen in clumps of growth, as if they had developed inside the colony. They stained neutrophilic by Giemsa's method (Pl. 1, fig. 4).

These appearances were seen only in media containing horse serum; they were absent from cultures enriched with human ascitic fluid or rabbit serum. Even after repeated cultivation on media without horse serum the P strains did not lose their ability to cause the characteristic effects when again grown in the presence of horse serum. There was no evidence that the changes in the medium represented the growth of another living agent apart from the pleuropneumonia-like organism. These changes appeared to result from the precipitation of some substance in the medium during growth; the exact nature of this substance was not determined, although it is probably protein. The spherical masses seen in fluid media did not dissolve in fat solvents or in weak acid.

Ledingham (1933) described what he regarded as crystal formation in both plate and fluid cultures of the organism of agalactia. His photograph of a surface culture shows black dots in the medium similar to those produced by the P strains; he does not mention, however, a film on the surface of the plate. I have been unable to examine a culture of agalactia in comparison with the P strains. Similar appearances on horse serum media were produced by a culture of the L 3 organism, but have not been seen in cultures of many strains of other members of the pleuropneumonia group that have been at one or other time investigated in this laboratory. They thus assist the identification of P strains.

Serology of P and S strains

Three P strains, differing antigenically, were investigated in detail. The results of agglutination and complement-fixation tests are shown in Table 1. Strains 2 and 14 were agglutinated to titres of 1/2048 and 1/8192 by their respective antisera, but neither was agglutinated by the heterologous antiserum. Strain 17 proved to be less specific. It was agglutinated by its homologous antiserum to a titre of 1/512 and also by antisera 2 and 14 to 1/8 and 1/32 respectively of their full titres. Antiserum 17 did not agglutinate strain 14, although it agglutinated strain 2 to full titre.

Table 2. *Results of absorbing Strain* 17 *rabbit antiserum*

	Strain 17 antiserum absorbed by strain			
Antigen	Nil	2	14	18
Strain 17	512	320	320	20
2	512	<20	—	—
14	<4	—	<20	—
18	512	—	—	<20

Although strain 17 was agglutinated by antisera 2 and 14, it proved to have a specific antigen by agglutinin-absorption tests (Table 2). After being absorbed twice with a heavy suspension of strain 2 the antiserum 17 no longer agglutinated strain 2 at a dilution of 1/20, but agglutinated strain 17 to the

Table 1. *Results of agglutination and complement-fixation tests with rabbit antisera*

Antigen	Type of organism	Strain 2 antiserum		Strain 14 antiserum		Strain 17 antiserum		Strain 15 antiserum	
		Agglutination titre	C.F.T. titre	Agglutination titre	C.F.T. titre	Agglutination titre	C.F.T. titre	Agglutination titre	C.F.T. titre
Strain 2	P	**2048**	**4096**	0	64	512	256	0	0
14	P	0	256	**8192**	**512**	0	8	0	0
17	P	256	1024	256	256	**512**	**2048**	0	0
15	S	0	0	0	0	0	0	**1024**	**512**
20	S	0	0	0	0	0	0	0	64
21	S	0	0	0	0	0	0	0	64
27	S	0	—	0	—	0	—	0	—
22	P	512	—	1024	—	512	—	0	—
23	P	0	—	8192	—	0	—	0	—
24	P	256	—	64	—	64	—	0	—
25	P	512	—	256	—	128	—	0	—
26	P	512	—	1024	—	512	—	0	—
28	P	256	—	256	—	512	—	0	—
29	P	128	—	32	—	0	—	0	—
Organism of bovine pleuropneumonia		0	0	0	0	0	0	4	0
Sewage A		0	0	0	0	0	0	0	512
Sewage B		0	0	0	0	0	0	0	128

Titre expressed as the reciprocal of the highest dilution at which there was agglutination or complete fixation of complement.
Lowest dilution tested for agglutination was 1/4 and by the C.F.T. 1/2.
— = no test.
Homologous titres are in heavy type.

original titre. Similarly, adsorption with strain 14 did not affect its ability to agglutinate strain 17. On the other hand, strain 18, isolated from another animal in the same herd from which strain 17 had been isolated, almost completely removed the agglutinins for strain 17.

Although strains 2 and 14 were antigenically distinct by the agglutination test, in the complement-fixation tests (C.F.T.) there were cross-reactions suggesting common antigens (Table 1). There was no correspondence between the magnitude of the agglutination and C.F.T. titres of the antisera for their homologous organisms. For instance, the agglutination titre of antiserum 14 was 16 times the C.F.T. titre, whereas antiserum 17 had an agglutination titre one-quarter of the C.F.T. titre. There may, therefore, possibly be differences between the antigens concerned in the two types of test. No soluble complement-fixing antigens were demonstrable in filtrates of cultures.

Seven other P strains were tested with the three P antisera (Table 1). Strain 23 was agglutinated to full titre by strain 14 antiserum only; strains 23 and 14 thus appeared to be of the same serological type, although they were isolated from animals in different herds. On the other hand, although strains 23 and 26 were isolated from two animals in the same herd they were apparently different antigenically. Strain 28 gave the same reactions as strain 17. Strain 29 was not agglutinated to significant titre by any of the antisera. Most of the others were agglutinated by all three antisera, usually only to partial titre. Two went to full titre with antiserum 17, but, since this antiserum tended to be non-specific their identity remained uncertain. The serology of the P strains is therefore complex. A number of different serological types appear to exist, but certain antigens are shared.

Four S strains were tested against an antiserum to the S strain 15. They were not agglutinated and did not fix complement with any of the P antisera; nor did antiserum 15 agglutinate or fix complement with any of the P strains (Table 1). The P and S types of organism are therefore serologically distinct. Antiserum 15, although agglutinating its homologous organism, did not agglutinate the other three S strains; however, with the two strains tested it did fix complement to about 1/10 of the full titre. Strains of the S type thus differ antigenically among themselves although certain antigens are shared.

It was not possible to compare the antigenic constitution of these organisms with that of all the other members of the pleuropneumonia group. However, certain serological relationships were explored, namely, those of the P strains with the organism of contagious bovine pleuropneumonia (the only other member of the group previously isolated from cattle) and of the S strains with the saprophytic organisms. The organism of bovine pleuropneumonia was not agglutinated by any of the antisera, except to a titre of 1/8 only by the S antiserum. The P strains are thus unrelated antigenically to the strain of the organism of bovine pleuropneumonia tested (no. 3278, National Collection of Type Cultures); of course this organism differs also in pathogenicity and does not produce the changes in media containing horse serum which are characteristic of P strains. Suspensions were prepared from two of Laidlaw & Elford's (1936) strains, Sewage A and B, which are antigenically different although

related; the third strain, Sewage C, a distinct serological type, was unfortunately no longer available. Klieneberger (1940), using the agglutination test, showed that Seiffert's strains were closely related to Sewage A. Neither the P antisera nor the S antiserum agglutinated the sewage organisms, but the S antiserum fixed complement to full titre with Sewage A and to partial titre with Sewage B.

Table 3. *Results of complement-fixation tests on sera from infected cattle*

	Serum + antigen + complement Serum dilutions					Serum + saline + complement Serum dilution
Animal	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{2}$
Cow 1	4	4	1	0	0	0
2	3	3	3	2	0	0
3	3	0	—	—	—	0
4	3	2	—	—	—	0
5	0	0	—	—	—	0
6	1	±	—	—	—	0
7	1	0	—	—	—	0
8	4	0	—	—	—	0
Bull 1	0	0	—	—	—	0
2	2	1	—	—	—	0
3	4	4	—	—	—	0
3 (repeat)	2	1	2	0	0	0

4 = complete fixation; 3 to 1 = degrees of incomplete fixation; ± = trace of fixation only; 0 = no fixation; — = no observation made.

Suspension of 'P' strain 17 used undiluted as antigen. Control tube containing antigen, saline and complement showed no fixation.

Serological tests of bovine sera. Sera from twelve infected cows and bulls from which pleuropneumonia-like organisms had been isolated were tested for agglutinins. The titres obtained did not differ significantly from those of sera from normal maiden heifers. A few of the cattle sera were tested against a suspension of the organism isolated from that particular animal or from another in the same herd.

The sera were also tested for complement-fixing antibodies. Using the P strain 17, three sera fixed complement completely in low dilutions and several others gave partial fixation (Table 3). Under these conditions there was no fixation of complement by eight sera from normal maiden heifers. The results were confirmed with other antigens. For the tests the sera were inactivated by heating to 62° for 30 min., and this temperature was found to be essential for the specificity of the test. When inactivated at 56° the infected sera showed a greater degree of fixation, but one of the control sera was also positive.

In an investigation of non-specific urethritis in man, Johnston & McEwin (1945) isolated pleuropneumonia-like organisms from the discharges of two patients. When saline suspensions of the organisms were injected intradermally both patients gave positive skin reactions. A group of eight cows and one bull, from which pleuropneumonia-like organisms had been isolated, was therefore skin-tested. Saline suspensions of a P strain, prepared from a culture in sloppy agar, were injected intradermally together with a control injection of material

similarly prepared from uninoculated culture medium. The skin reactions to the test inocula were no larger than those to the controls.

DISCUSSION

Two members of the pleuropneumonia group, provisionally called P and S, have been isolated from the genital tract of cattle. The cultural similarities and the presence of common complement-fixing antigens suggest the S strains to be closely related to the saprophytic organisms. S organisms are therefore unlikely to be pathogenic for cattle; they may inhabit the genital tract as commensals or, alternatively, live in the environment of the animals, from which they gain access to the vaginal discharges and semen as contaminants.

The P strains are of greater interest, and it is important to determine whether they are pathogenic for cattle. Investigations in progress, which suggest that these strains are pathogenic and capable of producing inflammatory changes in the genital tract, possibly predisposing to infertility, will be described elsewhere. In an examination of a small number of sera from infected animals for specific antibodies a failure to demonstrate agglutinins is recorded here, although a few of the sera appeared to fix complement specifically. The examination of a larger number of sera from infected and non-infected animals would be necessary before the specificity of the complement-fixation reaction could be assumed and the presence of complement-fixing antibodies adduced as evidence of actual infection by pathogenic organisms. Beveridge, Campbell & Lind (1946) found complement-fixing antibodies for a pleuropneumonia-like organism, isolated from the human genital tract, in 79 out of a group of 128 sera from cases of non-specific urethritis in man. Some doubt, however, as to the specificity of the test was raised by finding 7 out of 98 sera from healthy blood donors positive and 17 positive out of 24 from a group of cases of atypical pneumonia. Wallerstein, Vallee & Turner (1946) obtained positive complement fixation in two cases of Reiter's disease. Harkness & Henderson-Begg (1948), however, failed to demonstrate either complement-fixing antibodies or agglutinins in non-specific urethritis. Although information regarding the development of antibodies in animals infected by members of the pleuropneumonia group is incomplete, it would appear that agglutinins are demonstrable only irregularly even in contagious bovine pleuropneumonia (Sabin, 1941 *a*). Neutralizing antibodies were not found in the sera after recovery from certain pleuropneumonia-like infections of mice (Sabin, 1938) and rats (Woglom & Warren, 1938). The absence of agglutinins in the sera of cattle from whom P strains were isolated is therefore itself not evidence that the organisms were not pathogenic.

The demonstration that different serological types occur among the strains is of obvious importance in epidemiological and other studies; it is even possible that serological types may differ in their pathogenicity. Of greater significance was the finding that at least two different members of the pleuropneumonia group, one of which is probably non-pathogenic, can be isolated from the genital tract of cattle. Already there have been several examples of the importance of identifying a strain as a P or S type. For instance, at an early stage in the

investigation it was realized that most of the material examined and found to contain pleuropneumonia-like organisms came from herds under observation for breeding difficulties. Semen was therefore examined from the stock bull in a herd with a good breeding history. The finding in it of pleuropneumonia-like organisms was at first disappointing; only later was the strain recognized as an S type. On another occasion samples were received from two bulls in a herd where there were breeding troubles apparently of infective aetiology. Pleuropneumonia-like organisms were present in both, but not other bacteria of pathogenic significance. Each sample was inoculated into the uterus of a healthy maiden heifer at oestrus. One sample, which was shown to contain a P organism, produced inflammation of the genital tract in the inoculated animal. The other sample contained an S organism, and no ill-effect resulted from its inoculation. This experiment (together with others to be reported elsewhere) suggests that P strains may be pathogenic and S strains harmless. The isolation of both P and S organisms from animals in one herd emphasizes the need for the exact identification of every strain isolated.

P strains differed in the ease with which they grew in artificial culture; sometimes the colonies were poorly developed in the primary cultures and occasionally it was difficult or impossible to obtain subcultures. Some of the strains more difficult to grow came from animals in which there seemed to be greater evidence for the infectivity of the organism. Dienes, Ropes, Smith, Madoff & Bauer (1948) had rather similar experiences in their investigation of pleuropneumonia-like organisms from the human genital tract. Strains isolated from the male grew poorly and were often difficult to subculture, whereas strains were easier to isolate from the female. In the female it was evident that many strains, at least, were not pathogenic, but in the male there was evidence that the organisms were producing inflammation. It is therefore possible that the ability of a strain to grow in artificial culture may vary inversely with its pathogenicity. Growth in the primary culture may be aided by factors supplied in the heavy inoculum of vaginal mucus or semen, and successful maintenance in subculture may depend on the formation of non-pathogenic variants capable of growth in an artificial culture medium. If this is so the strains maintained and studied in the laboratory are not truly representative of those present in the genital tract.

Experience in cattle may help in assessing the significance of pleuropneumonia-like organisms in the human genital tract. These organisms have been isolated from patients with non-specific urethritis by a number of observers but their pathogenicity is still in doubt. One objection to regarding them as pathogenic has been their presence in the vagina of a high proportion of women, including those apparently healthy. This objection, however, appears to have little validity. At one time it might have been argued that, because streptococci could be found in the normal human throat, they could not be responsible for disease. Only as methods for bacteriological study advanced was it possible by morphological, cultural, biochemical and serological investigations, leading up to recognition of the 'M' substance, to identify virulent strains of pathogenic species, such as *Streptococcus pyogenes* Group A, among the whole group of

Gram-positive cocci growing in chains. No investigations have been made of strains of pleuropneumonia-like organism isolated from the human genital tract beyond those necessary to identify them as members of the pleuropneumonia group from their morphological and colonial appearances; no serological differentiation has been attempted. Sabin was the first to show that more than one species could be found in the same situation; he isolated several strains with completely different pathogenic properties from the upper respiratory tract of mice (Sabin, 1939; Sabin & Johnson, 1940). The finding of both a potentially pathogenic species and a saprophyte or commensal in the genital tract of cattle emphasizes the need for cultural and serological investigations of strains isolated from the human genital tract. Until these have been made a member of the pleuropneumonia group cannot be excluded as a possible cause of some of the infections in man diagnosed as non-specific urethritis.

Names have not been given to the two members of the pleuropneumonia group isolated from cattle. The major differences in their cultural properties and their antigenic dissimilarity suggest that they represent different species. Sabin (1941 *a*, *b*) has, in fact, suggested that the saprophytic pleuropneumonia-like organisms should be classified in a different family from those organisms needing media enriched with animal protein for artificial cultivation. Final agreement has, however, not been reached regarding the nomenclature of the pleuropneumonia group. It would seem impossible to decide on a classification until members of the group have been studied in greater detail; further information regarding their cultural, biochemical and serological properties and their nutritional requirements is particularly required. There is even much which is still controversial regarding their morphology, although this has been studied most. The term 'pleuropneumonia-like organism' has therefore been used, and the two species have been provisionally referred to by the letters P and S. Members of the group are sometimes called L organisms, but this designation has been avoided because, although shorter, it refers merely to the serial numbering of species isolated by one worker in this country.

Note. Investigations of the pleuropneumonia group have been hindered by the difficulty in maintaining strains, which has resulted in many, originally isolated in this country, being lost. Mr H. Proom has successfully preserved strains by freeze-drying and has undertaken to maintain a representative collection of these organisms at the Wellcome Research Laboratories, Beckenham, Kent, for the use of other investigators.

I am indebted to my colleagues, Mr S. L. Hignett and Mr J. L. Hancock, for carrying out the inoculations of cattle, and to Mr E. A. Jones and Mr E. Hitchcock, of the Wellcome Research Laboratories, Beckenham, for taking the photomicrographs.

REFERENCES

Beveridge, W. I. B., Campbell, A. D. & Lind, P. E. (1946). Pleuropneumonia-like organisms in cases of non-gonococcal urethritis in man and in normal female genitalia. *Med. J. Aust.* **1**, 179.

Dienes, L. (1947*a*). Isolation of pleuropneumonia-like organisms from pathological specimens with the aid of penicillin. *Proc. Soc. exp. Biol., N.Y.*, **64**, 165.

Dienes, L. (1947*b*). Isolation of pleuropneumonia-like organisms from *H. influenzae* with the aid of penicillin. *Proc. Soc. exp. Biol., N.Y.*, **64**, 166.

Fig. 1

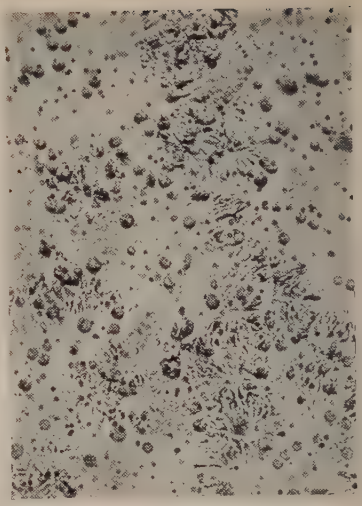


Fig. 2

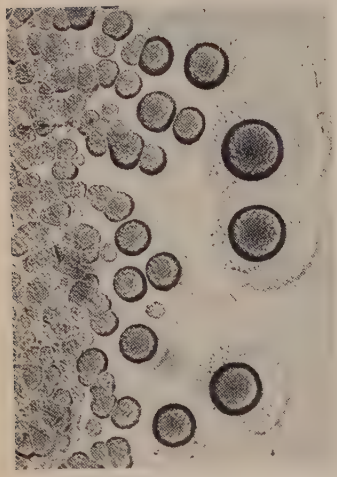


Fig. 3

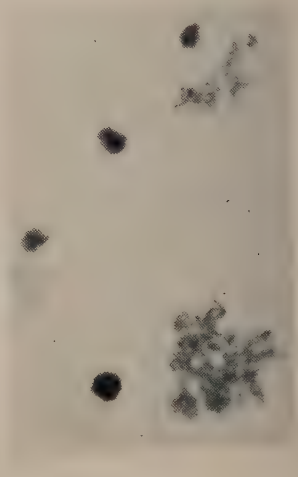


Fig. 4

D. G. FF. EDWARD—INVESTIGATION OF PLEUROPNEUMONIA-LIKE ORGANISMS. PLATE 1

Dienes, L., Ropes, M. W., Smith, W. E., Madoff, S. & Bauer, W. (1948). The role of pleuropneumonia-like organisms in genitourinary and joint diseases. *New Engl. J. Med.* **238**, 509, 563.

Edward, D. G. ff. (1947). A selective medium for pleuropneumonia-like organisms. *J. gen. Microbiol.* **1**, 238.

Edward, D. G. ff., Hancock, J. L. & Hignett, S. L. (1947). Isolation of pleuropneumonia-like organisms from the bovine genital tract. *Vet. Rec.* **59**, 329.

Harkness, A. H. & Henderson-Begg, A. (1948). The significance of pleuropneumonia-like or 'L' organisms in non-gonococcal urethritis, Reiter's disease and abacterial pyuria. *Brit. J. ven. Dis.* **24**, 50.

Johnston, G. A. W. & McEwin, J. (1945). Non-gonococcal arthritis. *Med. J. Aust.* **1**, 368.

Klieneberger, E. (1938). Pleuropneumonia-like organisms of diverse provenance: some results of an enquiry into methods of differentiation. *J. Hyg., Camb.*, **38**, 458.

Klieneberger, E. (1940). The pleuropneumonia-like organisms: further comparative studies and a descriptive account of recently discovered types. *J. Hyg., Camb.*, **40**, 204.

Laidlaw, P. P. & Elford, W. J. (1936). A new group of filtrable organisms. *Proc. Roy. Soc.* B, **120**, 292.

Ledingham, J. C. G. (1933). The growth phases of pleuropneumonia and agalactia on liquid and solid media. *J. Path. Bact.* **37**, 393.

Richardson, G. M. (1941). Preservation of liquid complement serum. *Lancet*, ii, 696.

Sabin, A. B. (1938). Isolation of a filtrable, transmissible agent with 'neurolytic' properties from toxoplasma-infected tissues. *Science*, **88**, 189.

Sabin, A. B. (1939). Mice as carriers of pathogenic pleuropneumonia-like micro-organisms. *Science*, **90**, 18.

Sabin, A. B. (1941*a*). The filtrable micro-organisms of the pleuropneumonia group. *Bact. Rev.* **5**, 1.

Sabin, A. B. (1941*b*). The filtrable micro-organisms of the pleuropneumonia group. (Appendix on classification and nomenclature.) *Bact. Rev.* **5**, 331.

Sabin, A. B. & Johnson, B. (1940). Pathogenic pleuropneumonia-like micro-organisms in tissues of normal mice and isolation of new immunological types. *Proc. Soc. exp. Biol., N.Y.*, **44**, 569.

Seiffert, G. (1937*a*). Ueber das Vorkommen filtrabler Mikroorganismen in der Natur und ihre Züchtbarkeit. *Zbl. Bakt.* (1 Abt., Orig.), **139**, 337.

Seiffert, G. (1937*b*). Filtrable Mikroorganismen in der freien Natur. *Zbl. Bakt.* (1 Abt., Orig.), **140**, 168.

Wallerstein, R., Vallee, B. & Turner, L. (1946). The possible relationship of pleuropneumonia-like organisms to Reiter's disease, rheumatoid arthritis and ulcerative colitis. *J. infect. Dis.* **79**, 134.

Woglom, W. H. & Warren, J. (1938). A pyogenic filtrable agent in the albino rat. *J. exp. Med.* **68**, 513.

EXPLANATION OF PLATE

Fig. 1. Cultures on horse serum agar plate. Upper portion seeded with 'P' strain; note white film covering bottom right portion of growth; colonies too small to be seen individually. Lower portion of plate seeded with 'S' strain; no film.

Fig. 2. Surface culture of 'P' strain seen by transmitted light; note small colonies of organism, in places covered by a crinkled film, also numerous small black dots. ×85.

Fig. 3. Surface culture of 'S' strain for comparison. Colonies large and typical of the pleuropneumonia group; no surface film or black dots in the medium. ×85.

Fig. 4. Film made from the deposit of a horse serum broth culture of a 'P' strain and stained by Giemsa. Two parts of the same field showing on the right two clumps of the organism and on the left characteristic bodies. ×2000.

(*Received* 23 *February* 1949)

The Influence of the Bacterial Environment on the Excystment of Amoebae from Soil

By LETTICE M. CRUMP
Department of Soil Microbiology, Rothamsted Experimental Station, Harpenden, Hertfordshire

SUMMARY: Cysts of two amoebae from soil were grown in single-cell cultures to test the effect upon excystment of the presence or absence of bacteria, age and type of bacteria, age of cyst, and the concentration of sodium chloride. Within the limits of the experiments excystment in one species was independent of the presence of bacteria, and unaffected by their age and type or by the age of cyst. The other species was more sensitive and could not excyst without living bacteria of a suitable type, and the cysts become less likely to develop with age. Excystment in both species was adversely affected by increasing the concentration of sodium chloride, distilled water giving the best results.

Living bacteria form the usual food of the small soil amoebae, but numerous references in the literature show that different bacteria have different effects on the amoebae (Frosch, 1897; Mouton, 1902; Oehler, 1916; Tsujitani, 1898; Cutler & Crump, 1927; Singh, 1941 *a*). There are also records of other organic particles being eaten, such as small yeasts (Mouton, 1902; Beijerinck, 1896; Oehler, 1916) and dead bacteria (Oehler, 1916; Tsujitani, 1898).

The suitability of a bacterial strain is usually judged either by the rate of reproduction of the amoebae (Cutler & Crump, 1927) or by the speed with which the bacteria disappear (Frosch, 1897; Singh, 1941 *a*). Thus single cells of *Hartmanella hyalina* showed a reproductive rate of 3·7 in the first 24 hr. of growth with one species of soil bacteria, and of 2·0 with another, when grown in otherwise identical conditions (Cutler & Crump, 1927), the time from isolation to excystment being approximately the same in both cases. Singh (1942), using mass cultures, divided his bacteria into groups according to whether they were readily and completely eaten, slowly and completely eaten, slowly and incompletely eaten, or not eaten at all. None of these criteria is wholly satisfactory since the amoebae are observed only up to the time of death or encystment and the ability of the cysts to excyst and carry on the race has not usually been studied. It is, however, well known that the percentage of viable cysts in cultures of Protozoa is very variable (Brand, 1923; Wolff, 1927; Cutler & Crump, 1927).

Many factors have been stated to induce cyst formation in various genera of Protozoa; among them are the following: lack of food (Beers, 1926; Brand, 1923; Johnson & Evans, 1940; Oehler, 1916; Singh, 1941 *b*; Wolff, 1927); crowding (Barker & Taylor, 1931); desiccation (Belar, 1921; Bodine, 1923; Brand, 1923; Kühn, 1915); gradual evaporation of medium (Garnjobst, 1928); accumulation of waste products of organism (Beers, 1926; Belar, 1921; Mast & Ibara, 1923; Stolte, 1922); metabolic products of bacteria (Belar, 1921; Kühn, 1915; Mouton, 1902); lack of oxygen (Brand, 1923; Stolte, 1922); alkalinity (Koffman, 1924; Ilowaisky, 1926); sudden fall in pH value (Darby, 1929); low tempera-

tures (Schmahl, 1926); optimum conditions for growth and reproduction (Kater & Burroughs, 1926); internal causes (Cutler & Crump, 1935; Ivanic, 1934).

Excystment is attributed to less varied causes on the whole; those most often cited are: addition of fresh liquid (Kühn, 1915); presence of bacteria (Brand, 1923; Frosch, 1897; Singh, 1941*b*); presence of oxygen (Brand, 1923); hypertonic solutions (Ilowaisky, 1926); acid pH of medium (Koffman, 1924); desiccation (Rhumbler, 1888; Ilowaisky, 1926; Wolff, 1927); neutral medium (Brand, 1923); organic infusions (Barker & Taylor, 1933).

It is difficult to discriminate among the various factors which may be present in the same culture at one time, and it is often impossible while varying one factor to keep the others constant; but it is clear that encystment and probably excystment in the Protozoa can be induced by a wide range of conditions, and probably even within one species there is not complete uniformity of behaviour.

MATERIAL AND METHODS

Two species of limax amoebae from soil (species 4 and species Z) were grown with two species of bacteria: an *Aerobacter* sp. and a Gram-negative short rod (4036) which is a denitrifying organism isolated from an arable Rothamsted soil. The *Aerobacter* sp. is recorded by Singh (1942) as being completely and readily eaten by all his amoebae; strain 4036 he found to be inedible (unpublished). After repeated subculturing with strain 4036 on non-nutrient agar plates both amoebae lived successfully on this organism.

The amoebae are of approximately the same size (15–20 μ), and the active forms are indistinguishable. Amoeba Z forms a cyst with a single wall; it grows rapidly and in a culture consisting of a circle of bacterial growth of about 1·5 cm. in diameter on a solid medium seeded with a few cysts of the amoebae at the centre, the amoebae usually clear the bacteria completely and have themselves encysted within 24 hr. The shortest period for excystment observed is 1·5 hr. from the time of isolation. Amoeba 4 forms a double-walled cyst and excystment takes place in two stages: first, the inner wall disappears and a small amoeba moves freely within the outer wall; then, in a successful case, the outer wall gives way and the amoeba emerges. If the outer wall remains impenetrable, as may often happen in unfavourable conditions, the amoeba dwindles away and ultimately dies. The growth and reproduction in this species is not so fast as it is in species Z; a mass culture with the *Aerobacter* sp. usually takes about 3 days to clear the bacteria and encyst. The most rapid excystment that has been observed in a single cyst is 3·0 hr.

Mass cultures of both amoebae were grown on non-nutrient agar with bacteria from 1 to 3 days old. 1·5 % agar was used with the addition of 0·5 % NaCl, 0·1 % K_2HPO_4 and 0·1 % KH_2PO_4; the pH was adjusted to 7·0. The plates were poured at 45° just before use. Single cysts were grown in counting chambers with the bacteria in sterilized water unless otherwise stated. Since actual excystment is not a guarantee of the animal's vigour, excysted amoebae were kept for 24 hr. so that the reproductive rate for that period was known. All cultures were incubated at 25°.

RESULTS

The effect on excystment of varying five of the conditions to which the cysts were exposed was as follows.

Amount of sodium chloride in medium. The salt concentration of the solution to which the cysts were exposed affected excystment; more amoebae emerged at the lower concentrations (Table 1), which accords with the findings of Beers (1945) in *Tillina magna* and Garnjobst (1928) in *Euplotes taylori*. Relatively few amoebae succeeded in emerging at NaCl concentrations greater than 0·75 %; species 4 was more sensitive than Z.

Table 1. *Effect of salinity on excystment in 24 hr. in presence of* Aerobacter *sp.*

		NaCl (%)					
	Amoeba	0	0·25	0·5	0·75	1·0	1·5
No. of cysts observed	Z	34	24	47	25	48	54
Excystment (%)		97·1	87·5	82·9	76·0	29·2	14·8
No. of cysts observed	4	20	35	39	37	21	21
Excystment (%)		95·0	76·5	54·5	44·2	11·1	0

Age of cysts. Within the range of the ages tested (Table 2) the cysts of amoeba Z showed no significant differences in behaviour, but some of the cysts of amoeba 4 became non-viable with age.

Table 2. *Percentage excystment after 24 hr. of single cysts transferred from cultures fed with* Aerobacter *sp. into water containing* Aerobacter *sp.*

		Age of parent culture (days)						
	Amoeba	1–3	4–6	7–9	10–12	13–15	16–18	19–21
No. of cysts observed	Z	80	44	69	153	89	52	81
Excystment (%)		100	100	97·1	93·4	94·3	86·5	93·8
No. of cysts observed	4	6	22	57	37	64	15	—
Excystment (%)		100	95·4	85·9	89·2	70·3	60·0	—

Presence and absence of bacteria. Cysts of amoeba Z excysted readily in water without bacteria, but those of species 4 excysted very rarely unless living bacteria were present (Table 3). No excystment was observed in species 4 in the absence of living bacteria when cysts were transferred into the supernatant fluid obtained by centrifuging suspensions made from 24 hr. old cultures of either of the two bacteria in water.

Table 3. *Percentage excystment after 24 hr. of single cysts, from cultures grown with* Aerobacter *sp., placed in water with and without* Aerobacter *sp.*

	Amoeba	With *Aerobacter* sp.	Without *Aerobacter* sp.
No. of cysts observed	Z	114	81
Excystment (%)		92·1	93·8
No. of cysts observed	4	86	85
Excystment (%)		82·4	0

Age of food supply. In all experiments the numbers of bacteria were very much in excess of the requirements for inducing a maximum reproductive rate, so that even when cysts were transferred to an old culture containing numbers of dead and moribund bacteria there were plenty of living bacteria available. Although in some of the age groups there were not many observations (Table 4) it seems clear that within wide limits the age of the bacteria

Table 4. *Percentage excystment after 24 hr. in cysts transferred from cultures fed with* Aerobacter *sp. into water containing* Aerobacter *sp. cultures of different ages*

		Age of *Aerobacter* sp. culture (days)						
	Amoeba	1–3	4–6	7–9	10–12	13–15	16–18	18–21
No. of cysts observed	Z	195	109	14	35	13	26	30
Excystment (%)		96·9	89·0	85·7	77·2	84·6	96·1	80·0
No. of cysts observed	4	109	53	14	—	—	—	21
Excystment (%)		89·0	84·9	92·8	—	—	—	95·2

supplied is without effect on the successful excystment of the amoebae. In species 4, however, the age of the bacteria in the medium influences the length of time between isolation and excystment; a few records only have been made, but the result is clear (Table 5).

Table 5. *Time of excystment of species 4 with* Aerobacter *sp. of different ages*

	Age of *Aerobacter* sp. (days)				
	1	2	4	9	More than 9
No. of cysts observed	22	19	16	16	16
Average time of excystment	3 hr. 20 min.	3 hr. 40 min.	4 hr. 10 min.	4 hr. 25 min.	6 hr. 40 min.

Strain of bacterium to which cysts were exposed. Cysts formed in cultures with the *Aerobacter* sp. and 4036 were grown with both types of bacteria. Species Z is very little affected by the change in bacteria, but in species 4 there is a difference in response (Table 6).

Table 6. *Percentage of excystment after 24 hr. for amoebae Z and 4 transferred to water*

		Bacteria in medium	
	Amoeba	*Aerobacter* sp.	4036
Cysts formed with *Aerobacter* sp.:			
No. of cysts observed	4	263	219
Excystment (%)		91·2	32·3
No. of cysts observed	Z	223	60
Excystment (%)		94·5	96·6
Cysts formed with 4036:			
No. of cysts observed	4	201	179
Excystment (%)		98·6	45·5
No. of cysts observed	Z	80	80
Excystment (%)		95·0	95·0

CONCLUSIONS

Since species 4 cannot excyst without bacteria it seems that these two strains of bacteria produce some material inducing excystment in this amoeba (Thimann & Barker, 1934), but that the product of the *Aerobacter* sp. is the more efficacious. The unsuccessful attempts already recorded to induce excystment by placing the cysts in culture fluid in which *Aerobacter* sp. or 4036 had grown, but from which they had been removed by centrifuging, suggest that the substances concerned may be so transient that they disappear as fast as they are formed, and only when there are living bacteria in the medium is enough of the stimulating material present to act successfully on the cysts.

It can be argued either that in the presence of strain 4036 the amoebae produce a cyst wall which is relatively weak, or that the amoebae which ultimately survive on 4036 are a tougher race and give rise to a higher percentage of viable cysts.

Cysts of species 4 with the *Aerobacter* sp. showed a consistently high rate of excystment whether they had been formed in the presence of *Aerobacter* or of 4036 (Table 6), but when such cysts were grown with 4036 there was a marked decrease in the numbers which excysted. Cysts formed in cultures fed with 4036 showed a significantly higher rate of excystment than those from an *Aerobacter* parentage; where the cysts were grown with *Aerobacter* the difference was not so marked, 91·2 and 98·6 %, though this difference is significant at the 5 % level, but in isolations with 4036 the difference was as between 32·3 and 45·5 %, which is highly significant.

I wish to thank Dr H. G. Thornton, F.R.S., and Dr B. N. Singh for suggestions. I am indebted to Mr J. H. A. Dunwoody for determining the significance of the figures given in Table 6.

REFERENCES

Barker, H. A. & Taylor, C. V. (1931). A study of the conditions of encystment of *Colpoda cucullus*. *Physiol. Zoöl.* **4**, 620.

Barker, H. A. & Taylor, C. V. (1933). Studies on the excystment of *Colpoda cucullus*. *Physiol. Zoöl.* **6**, 127.

Beers, C. D. (1926). The life-cycle in the ciliate *Didinium nasutum* with reference to encystment. *J. Morph.* **42**, 1.

Beers, C. D. (1945). Some factors affecting excystment in the ciliate *Tillina magna*. *Physiol. Zoöl.* **18**, 80.

Beijerinck, M. W. (1896). Kulturversuche mit Amoeben auf festen substrate. *Zbl. Bakt.* (1 Abt., Orig.), **19**, 257.

Belar, K. (1921). Untersuchungen ueber Thecamoeben der *Chlamydophrys*-Gruppe. *Arch. Protistenk.* **43**, 287.

Bodine, J. H. (1923). Excystation of *Colpoda cucullus*. *J. exp. Zool.* **37**, 115.

Brand, Th. v. (1923). Encystierung bei *Vorticella microstoma* und hypotrichen Infusorien. *Arch. Protistenk.* **47**, 59.

Cutler, D. W. & Crump, L. M. (1927). The qualitative and quantitative effects of food on the growth of a soil amoeba (*Hartmanella hyalina*). *Brit. J. exp. Biol.* **5**, 155.

Cutler, D. W. & Crump, L. M. (1935). The effect of bacterial products on amoebic growth. *Brit. J. exp. Biol.* **12**, 52.

Darby, H. H. (1929). The effect of the hydrogen-ion concentration on the sequence of protozoan forms. *Arch. Protistenk.* **65**, 1.

Frosch, P. (1897). Zur Frage der Reinzuchtung der Amoeben. *Zbl. Bakt.* (1 Abt.), **21**, 926.

Garnjobst, L. (1928). Induced encystment and excystment in *Euplotes taylori*, sp.nov. *Physiol. Zoöl.* **1**, 561.

Ilowaisky, S. A. (1926). Material zum Studium der Cysten der Hypotrichen. *Arch. Protistenk.* **54**, 92.

Ivanic, M. (1934). Ueber die Ruhestadienbildung und die damit am Kernapparate verbundenen Veranderungen bei *Lionotus cygnus* O.F.M. (*Amphileptus anser* Ehrbg.). *Zool. Anz.* **108**, 17.

Johnson, W. H. & Evans, F. R. (1940). Environmental factors affecting encystment in *Woodruffia metabolica*. *Physiol. Zoöl.* **13**, 1.

Kater, J. McA. & Burroughs, R. D. (1926). The cause and nature of encystment in *Polytomella citri*. *Biol. Bull. Woods Hole*, **50**, 38.

Koffman, M. (1924). Ueber die Bedeutung der Wasserstoffionenkonzentration für die Encystierung bei einigen Ciliatenarten. *Arch. mikr. Anat.* **103**, 168.

Kühn, A. (1915). Ueber Bau, Teilung und Encystierung von *Bodo edax* Klebs. *Arch. Protistenk.* **36**, 212.

Mast, S. O. & Ibara, Y. (1923). The effect of temperature, food and the age of the culture on the encystment of *Didinium nasutum*. *Biol. Bull. Woods Hole*, **45**, 105.

Mouton, H. (1902). Recherches sur la digestion chez les amibes et sur leur diastases intracellulaire. *Ann. Inst. Pasteur*, **16**, 457.

Oehler, R. (1916). Amobenzucht auf reinem Boden. *Arch. Protistenk.* **37**, 175.

Rhumbler, L. (1888). Die verschiedenen Cystenbildungen und die Entwicklungsgeschichte der holotrichen Infusoriengattung Colpoda. *Z. wiss. Zool.* **46**, 449.

Schmahl, O. (1926). Die Neubildung des Peristoms bei der Teilung von *Bursaria truncatella*. *Arch. Protistenk.* **54**, 359.

Singh, B. N. (1941*a*). Selectivity in bacterial food by soil amoebae in pure mixed culture and in sterilized soil. *Ann. appl. Biol.* **27**, 52.

Singh, B. N. (1941*b*). The influence of different bacterial food supplies on the rate of reproduction in *Colpoda steinii*, and the factors influencing encystation. *Ann. appl. Biol.* **27**, 65.

Singh, B. N. (1942). Selection and bacterial food by soil flagellates and amoebae. *Ann. appl. Biol.* **29**, 18.

Stolte, A. H. (1922). Verlauf, Ursachen und Bedeutung der Encystierung bei *Blepharisma*. *Verh. dtsch. Zool. Ges.* **27**, 79.

Thimann, K. V. & Barker, H. A. (1934). Studies on the excystment of *Colpoda cucullus*. II. The action of the excystment inducing substance. *J. exp. Zool.* **69**, 37.

Tsujitani, J. (1898). Ueber die Reinkulturen der Amoeben. *Zbl. Bakt.* (1 Abt.), **24**, 666.

Wolff, E. (1927). Un facteur de l'enkystment des Amibes d'eau douce. Prolongation experimentale de la periode vegetative. *C.R. Soc. Biol., Paris*, **96**, 636.

(*Received* 16 *March* 1949)

The Relation of Toxicity and Enzyme Activity in the Lecithinases of *Clostridium bifermentans* and *Clostridium welchii*

By ELLEN M. MILES and A. A. MILES
National Institute for Medical Research, Hampstead, London

SUMMARY: Crude preparations of the lecithinase of *Clostridium bifermentans* are lethal for mice. Their toxicity, haemolytic activity, and the power of liberating acid-soluble phosphorus from lecithin are neutralized by *Cl. welchii* α-antitoxin; in terms of lethal doses of *Cl. welchii* α-toxin and the *bifermentans* lecithinase, α-antitoxin is less than one five-hundredth as effective against the *bifermentans* lecithinase as against the α-toxin.

In terms of *in vitro* lecithinase activity in standard conditions, equipotent amounts of lecithinase from three different strains of *Cl. bifermentans* were respectively 9, 60 and 75 times less toxic than the corresponding amount of *Cl. welchii* lecithinase. The toxicity of the *Cl. bifermentans* lecithinase therefore is determined, not only by its lecithinase activity, but by unknown factors that vary from strain to strain of the bacillus. These factors are not likely to be contaminating substances with a synergic or inhibitory effect, since the differences in toxicity were maintained in several preparations from each of the three strains studied.

The analysis of the two antigenically related lecithinases from *Cl. welchii* and *Cl. bifermentans* provided no positive evidence against the hypothesis that the α-toxin of *Cl. welchii* is identical with the lecithinase.

In our last paper (Miles & Miles, 1947) we established that *Clostridium bifermentans* produced an enzyme which appeared to be a lecithinase C, and was antigenically related to the α-toxin of *Cl. welchii*. The potency of the α-toxin is proportional to its power to hydrolyse lecithovitellin with the liberation of acid-soluble phosphorus (Macfarlane & Knight, 1941). At the time, we had not enough *bifermentans* lecithinase for measuring its toxicity in terms of its phosphate-liberating power. In this paper we compare the *welchii* lecithinase (WL) with toxic preparations of the *bifermentans* lecithinase (BL).

MATERIALS AND METHODS

Preparation of WL and BL. As before, the WL (*welchii* lecithinase) preparations consisted of a crude dried toxin, 'Welchpool' (abbreviated to W'pool), and a glycerinated toxin, 290. The chief difficulty of preparing potent BL (*bifermentans* lecithinase) is the poor yield of toxic material from culture; in optimum conditions, about 100 ml. of culture yield one minimum lethal dose for 20 g. mice. We found no better medium than Macfarlane & Knight's (1941) glucose peptone muscle-extract medium; and BL was prepared from it as follows. Six litres of medium in three Winchester quart bottles filled almost to the top were warmed to 37° and seeded with *c.* 20 ml. of an overnight anaerobic culture of *C. bifermentans* in the same medium. After 18 hr. incubation the turbid cultures were filtered successively through a Seitz clearing disk and a Berkfeld candle. The filtrate was held at 5–7°, and concentrated *c.* 40-fold in

cellophane bags under a pressure of $\frac{2}{3}$ atm., and the concentrate dialysed at room temperature for 18 hr. against running tap water. The whole process took $2\frac{1}{2}$–3 days. The concentrate was freeze-dried in flasks and held at $-5°$. Four such dried preparations were then pooled in a minimum amount of water and the pool freeze-dried. The resulting fluffy, pale yellow, readily water-soluble substance was distributed in 0·5 g. lots into ampoules, dried over P_2O_5, sealed in N_2 and stored in the dark at $-5°$. The yield from 24 l. was *c.* 9 g. In all, over nine large batches were made. The material kept indefinitely in the cold under N_2, but lost over 50 % of its lecithinase activity in a fortnight when exposed to air for the purposes of sampling, and held in the intervals at room temperature over P_2O_5 *in vacuo*. Glycerinated solutions were more stable.

The material consisted of all the soluble large-molecular products of *Cl. bifermentans*, including various enzymes (Dr E. S. Duthie, for example, found powerful proteinase activity in our preparations). Our attempts to fractionate with ammonium sulphate or acetone, at room temperature and in the cold, and at various pH values, were unsuccessful, since a great deal of lecithinase was destroyed in the process. We therefore used the crude material for our experiments, relying on the specific neutralization of BL activity by *Cl. welchii* antitoxin to distinguish effects due to BL itself from those due to impurities in the preparation. The validity of this antitoxin control depends on the assumption that *Cl. welchii* and *Cl. bifermentans* are serologically related only in respect of their lecithinases.

With subculture, strains partly lost their power to produce BL in liquid media. Three strains of *Cl. bifermentans*, namely, M 58*e* used in our previous work, and two other wound strains M 10*g* and A 250*c*, were plated on human-serum agar. Colonies producing a large Nagler reaction were grown for 18 hr. in cooked-meat medium, and the centrifuged deposit of growth distributed into ampoules and preserved by Stamp's (1947) gelatin-ascorbic acid method, to serve as stable, uniform starting material for each batch.

In spite of its containing so many bacterial products, the final material was remarkably non-toxic. Only BL from M 58*e* was toxic, and its toxicity was entirely neutralized by *Cl. welchii* antitoxin. Material from the other two strains could be given intravenously to 20 g. mice in doses as large as 50 mg. without producing more than half an hour's discomfort and staring coat, 30 min. after injection.

Measurement of enzyme activity. The lecithinase activity of WL and BL solutions was measured (*a*) by titrating serial dilutions against yolk extract, expressing the result as arbitrary BL or WL units/ml. (Miles & Miles, 1947), and (*b*) by estimating the acid-soluble P liberated from fine suspensions of crude hen-egg lecithin. Crude lecithin was prepared by the method of Macfarlane & Knight (1941), stored under N_2 at 2° and used within 7–10 days. For enzyme titrations it was suspended in water, 20 mg./ml. The maximum acid-soluble P liberable from 1 mg. of successive preparations varied between 0·028 and 0·034 mg. Solutions of the crude WL and BL had a pH between 6·8 and 7·8, and were readily buffered by M/15–20 buffers. Accordingly, the reaction mixture usually consisted of 1 vol. each of lecithin suspension, lecithinase solution, and either

2 vol. M/5 Palitzsch's borate buffer (Palitzsch, 1915) pH 7·5, or 1 vol. buffer and 1 vol. anti-enzymic serum. Although BL has an optimum activity in the region of pH 5, the higher pH was used as a routine, partly because at pH 5 the lecithin suspensions were slightly unstable and filtration of trichloroacetic acid precipitates was difficult; but mainly because for our purpose it was more important to measure enzyme activity at pH values similar to those of animal tissues. For titrations of WL, $CaCl_2$ was added to make a 0·0133 M solution.

The hydrolyses were carried out in test-tubes immersed in a 37° bath so that half the fluid column was above the water-level, to promote continuous mixing by convection. All solutions were warmed to 37° before mixing. Acid-soluble P was estimated by the method of King (1947) with slight modifications. Four ml. of reaction mixture were treated with 2 ml. of trichloroacetic acid for 5–10 min. and filtered. The filtrate was rather resistant to oxidation; 0·5 ml. were evaporated to dryness with one drop of a saturated solution of Na_2CO_3 in a 15 ml. volumetric flask, and digested with 60 % perchloric acid. Digestion was usually complete 5 min. after the disappearance of colour. The molybdate colour was estimated against phosphate standards in an EEL (Evans Electroselenium Ltd., Harlow, Essex) photoelectric colorimeter. With an EEL 'tricolour red' screen the ammeter readings were strictly proportional to concentrations of P.

We depended on the added trichloroacetic acid to stop enzyme activity at the moment of sampling. Blanks were made by adding the trichloroacetic acid to the mixture of lecithin and buffer, and immediately afterwards adding the lecithinase solution. In definitive tests, samples were taken from the reaction mixture in triplicate, and estimated separately.

Haemolysin tests. Haemolysis was measured on red cells thrice washed in 0·85 % saline, and suspended in saline, 5 % (v/v) of packed centrifuged deposit. Falling dilutions of BL and WL were titrated, and approximate 50 % end-points (HD 50) determined by comparing haemoglobin liberated after 1 hr. at 37° with that in comparison tubes containing the same red-cell suspension lysed with saponin, and diluted to contain 90, 70, 50, 30 and 10 % of the original haemoglobin.

Toxicity tests. WL and BL preparations were injected intravenously in 18–22 g. mice; 0·2 ml. of preparation with 0·2 ml. of either saline or antiserum.

RESULTS

The hydrolysis of crude lecithin by BL

The comparison of hydrolytic activity. The curves for the liberation of acid-soluble P from lecithin by a given concentration of BL after varying periods of time ('time-P' curves; Fig. 1) and by varying concentrations of BL after a fixed time ('dose-P' curves; Fig. 2) are similar. In both, the lower portion of each curve is approximately linear, and both tend to become horizontal at the level of the maximum amount of liberable P for the preparation of lecithin used. The method of varying the enzyme concentrations was preferred, because it corresponds to the method of Macfarlane & Knight, and was technically less

laborious when comparisons of enzyme potency were made. Moreover, the dose-P curves are more regular than the time-P curves, with a longer and more definite part of the curve that is approximately straight. Owing to the relatively feeble potency of BL preparations, 30 min. was chosen as the

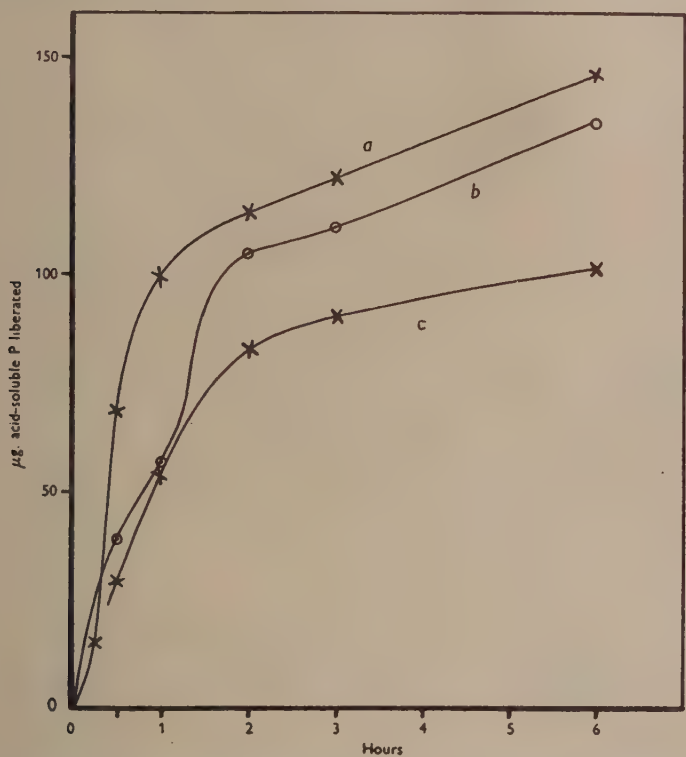


Fig. 1. The rate of liberation of acid-soluble P at 37°, pH 7·5, from suspensions of crude lecithin containing 0·140–0·160 mg. liberable P, by the following preparation of BL: (*a*) 5 mg. of M10*g*, BL13; (*b*) 5 mg. of A250*c*, BL18 and (*c*) 2·5 mg. of M58*e*, BL19.

standard time, instead of the 15 min. of Macfarlane & Knight, so as to have measurable amounts of P and at the same time economize in the use of BL preparations. There was some variation in repeated tests with different batches of lecithin; all comparative estimates of lecithinase potency were made therefore on one batch. Three concentrations were tested, in a range chosen to fall on the straight part of the curve. The linearity, however, of any substantial length of the P-liberation curve was difficult to establish, and for definitive estimates of potency a regression line was fitted to the three points plotted as log concentrations of the enzyme, against P liberated. In Fig. 3, the results for the two WL preparations and BL preparation, two from strain M58*e*, and one each from M10*g* and A250*c* are summarized. Each point is the mean of estimates

made on three samples taken from the same reaction mixture. The line was fitted and an analysis of variance made by the method of Fisher (1948). The variance of these means is very small, as the following typical figures for BL M10*g* show:

Dose (mg.)	*Acid-soluble* P *liberated (mg.)*
5·00	0·067, 0·068, 0·069
2·50	0·039, 0·041, 0·039
1·25	0·018, 0·019, 0·017

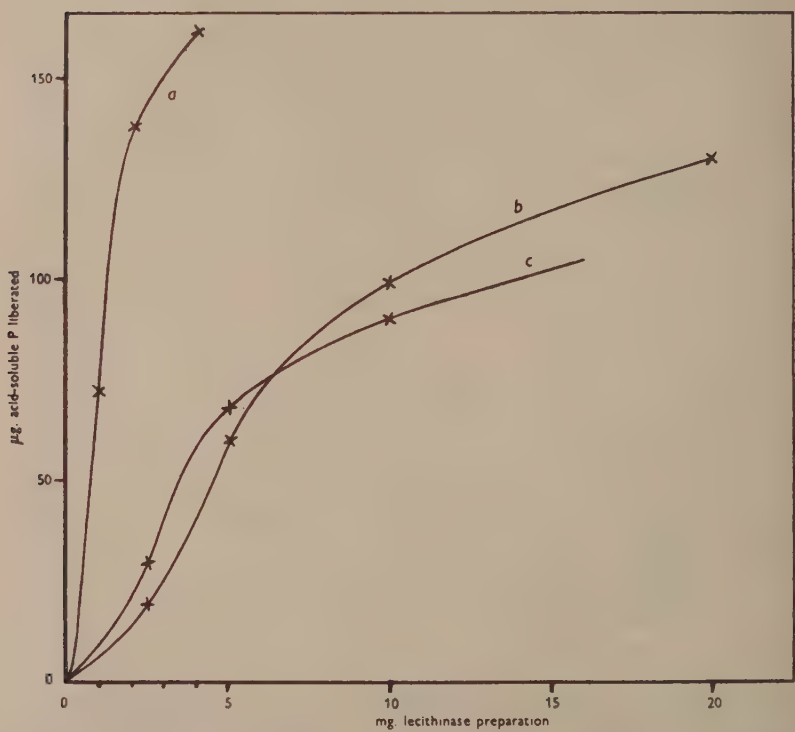


Fig. 2. The liberation of acid-soluble P after 30 min. at 37°, pH 7·5, from suspensions of crude lecithin containing 0·140–0·170 mg. liberable P, by graded amounts of various lecithinase preparations (*a*) WL W'pool; (*b*) and (*c*) A250*c*, BL18, on two batches of lecithin.

The weights of the means are very large, and hence all the departures from linearity, obvious in Fig. 3, are significant. It is therefore impossible from these figures to decide whether the most convenient linear relation is in fact given by plotting log concentration of enzyme against weight of P liberated, either for WL or BL; though it is certainly more convenient than that of concentration of enzyme against weight of P. We estimated the standard dose of enzyme by calculating from the fitted line the weight of lecithinase preparation liberating 50 μg. acid-soluble P in 30 min. at 37°. This we have symbolized as

DL50P, the dose liberating 50 μg. P (the symbol is awkward, but was so devised to distinguish it from analogues of the established LD50 notation of Trevan (1927), with which it is not strictly analogous). The DL50P of the six preparations are listed in Table 1, with the slopes of the regression lines. By regression analysis, the four slopes for the BL preparations proved to be

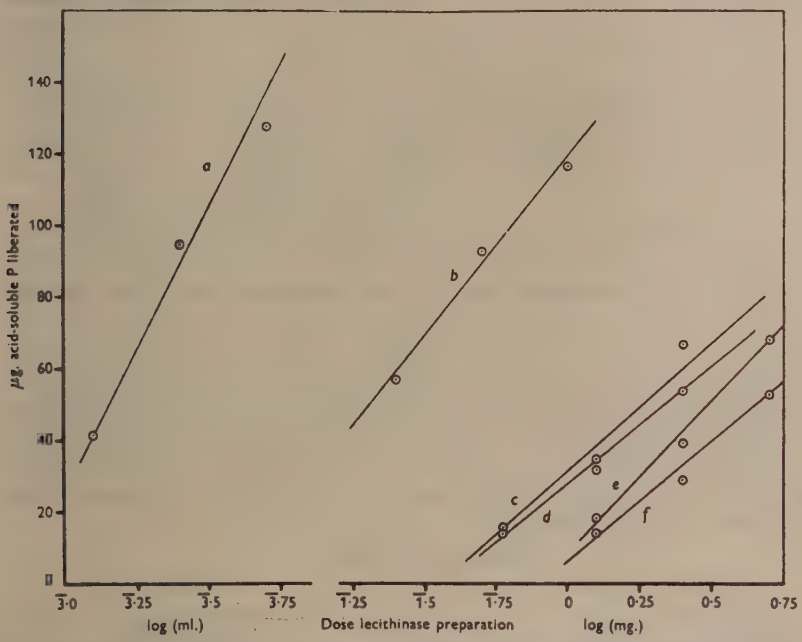


Fig. 3. A comparative estimate of phosphate liberation from one batch of lecithin by BL and WL preparations, the amount of P liberated being plotted against log dose enzyme, and regression lines fitted (see Table 5): (*a*) WL290; (*b*) WL W'pool; (*c*) and (*d*) BL20; (*e*) BL18; (*f*) BL17.

homogeneous and had a common slope of 0·0718; thus providing evidence that the four enzyme preparations behaved in the same way. The two slopes for WL differ significantly from the BL slopes; and from one another, though to a lesser extent. It follows that though the ratios of the DL50P indicate the relative potencies for a wide range of concentrations of the BL preparations, ratios of WL to BL potencies, or of WL potencies to one another, are strictly valid only for the DL50P itself.

The DL50P of successive batches of BL from one strain was of the same order of size, varying much as the two batches of M58*e* recorded in Table 1, i.e. within about 20% of each other. Preparations from strains M10*g* and A250*c* were always weaker in lecithinase than those from M58*e*.

The nature of the hydrolysis. Additional evidence that the acid-soluble P compound liberated from lecithin by BL is phosphorylcholine was obtained by a chromatographic analysis of the breakdown products. BL and WL were added

in amounts calculated to liberate most of the acid-soluble P present in the lecithin; each was added to 40 mg. crude lecithin in 8 ml. water, and held at 37° for 30 min. The mixtures were then treated with trichloroacetic acid and filtered. Our colleague, Dr T. S. Work, devised and carried out the analysis.

Table 1. *The doses of BL and WL preparations liberating* 50 *μg. acid-soluble* P (*DL* 50 *P*) *from crude lecithin at* 37° *under standard conditions; and the slopes of the log dose/phosphate-liberation regression line*

Bacterium	Batch	DL 50 P	Slope of regression line
Cl. bifermentans: A 250*c*	BL 17	4·63 mg.	0·0664
M 10*g*	BL 18	3·13 mg.	0·0830
M 58*e*	BL 19	1·80 mg.	0·0747
	BL 20	2·24 mg.	0·0653
Cl. welchii	WL W'pool	0·203 mg.	0·0997
	WL 290	0·00143 ml.	0·1583

The filtrates were extracted with an equal volume of $CHCl_3$. The extracted aqueous solutions were filtered and evaporated to dryness *in vacuo*. The residues were dissolved in 0·2 ml. water; 0·1 ml. was placed directly on no. 4 Whatman paper, the remaining 0·1 ml. after hydrolysis with 5N-HCl for 16 hr. at 110°, to split the phosphoryl radical from any phosphorylcholine present. The four samples, two of unhydrolysed and two of acid-hydrolysed material, were run in phenol against authentic samples of choline. After drying the paper was sprayed with Kraut's reagent (von Oettingen & Bowman, 1933), which showed spots as follows:

Lecithin split by	Acid-soluble material hydrolysed by	Choline
WL W'pool	Nil	Trace
	HCl	Large quantity
BL 17	Nil	Nil
	HCl	Large quantity

It is clear that BL, like WL, liberates an acid-soluble compound, which in turn liberates choline on acid hydrolysis; in conjunction with the fact that BL liberates acid-soluble P, this strongly suggests that the compound is phosphorylcholine, and confirms our original classification of the *bifermentans* enzyme as a lecithinase C.

Nomenclature of the enzyme. In so classifying the enzyme we followed Macfarlane & Knight's (1941) procedure with the α-toxin of *Cl. welchii*. Bard & McClung (1948), however, object to this designation for enzymes splitting phosphorylcholine from lecithin, on the grounds that Contardi & Ercoli (1933*a*) had already used the label C for a hypothetical enzyme yielding choline from lecithin, and suggest that the new enzyme should have been named 'Lecithinase D'.

It is true that in two papers (1933*a*, *b*) Contardi & Ercoli mentioned four theoretically possible enzymes attacking different linkages in the lecithin molecule, of which lecithinases A (giving lysolecithin) and B (giving glycerophosphorylcholine and two fatty acids) were already known to exist, C was

postulated as yielding choline and D as yielding phosphorylcholine; but at that time (1933*b*) these authors also pointed out that there was no evidence that types C and D existed. In a subsequent review, Belfanti, Contardi & Ercoli (1936) designated only lecithinases A and B, and no other possible lecithinases, such as C and D, were mentioned.

We think it is proper therefore to use Macfarlane & Knight's designation 'lecithinase C' for an enzyme splitting phosphorylcholine from lecithin, on the following grounds:

(1) The designation uses the next available letter for a properly characterized enzyme. Earlier use of a letter for a hypothetical enzyme, to which the evidence did not point unequivocally, in a scheme of classification that was neither generally accepted, nor maintained by its proposers, cannot be held as a valid pre-emption of that letter.

(2) The designation 'lecithinase C' for the *Cl. welchii* enzyme and other enzymes of this type has been used in the literature since its introduction in 1941, and to change it would confuse the situation unnecessarily.

Toxicity tests

Table 2 summarizes a number of toxicity tests, typical of many more. Exps. 1–6 show that in doses of 40–50 mg., various batches of BL from M10*g* were slightly toxic for mice, and that the toxicity was neutralized by *welchii*

Table 2. *The toxicity of BL and WL preparations, given intravenously in* 18–22 *g. mice*

Exp. no.	Strain and batch of lecithinase		Dose (mg.)	No. of mice dying/group: Without α-antitoxin	No. of mice dying/group: With α-antitoxin
1–3	M10*g*	BL9	40	0/3, 1/3, 1/3	—
4, 5		BL13	40	0/3, 0/3	—
6		BL18	50	2/8	0/8
7, 8	A250*c*	BL10	50	0/3, 0/3	—
9		BL12	50	0/5	—
10		BL17	50	1/9	2/9
11	M58*e*	BL19	40	5/5	1/5
			20	4/5	0/5
			10	3/5	0/5
			5	1/5	0/5
12	M58*e*	BL19	20	(20/20)*	
			10	7/20 (LD50 15·01 mg.	
			5	3/20	±0·232)
		WL W'pool	0·25	(20/20)*	
			0·20	19/20 (LD50 0·15 mg.	
			0·15	10/20	±0·0021)
		WL290	0·00132†	15/20	
			0·00110†	3/20 (LD50 0·00122 ml.	
			0·00090†	2/20	±·0000016)

* Omitted in calculation of LD50.
† ml.

antitoxin. BL from A 250*c* was less specifically toxic, though there was some toxicity not neutralized by an antitoxin (Exps. 7–10). The toxicity of BL 19, from M 58*e*, was considerable and was neutralized by antitoxin (Exp. 11). The toxicity of BL 19 and of the two WL preparations was determined in a single experiment (12), and the LD 50 calculated for each by the method of Gaddum (1933), with the results shown in the table.

Non-specific deaths usually occurred within ½ hr. of injection. Specific BL deaths took place between 2 and 24 hr. and resembled those due to WL; the more rapidly dying animals discharged blood-stained watery fluid from nose and urethra, and died with slowed respiration and staring coat. Post-mortem, the bladder was filled with blood-stained fluid and the muscles were stained a pale yellow-brown, presumably due to haemolysis. The degree of intravascular haemolysis however, was not large during life, for in mice given an intravenous dose of BL sufficient to kill in 2–4 hr., only from 2–5 % of the total haemoglobin in the blood had been liberated from the red cells in 1 hr. It is, of course, possible that BL modified the remaining red cells so that they became more susceptible to destruction in the tissues. No intravenous haemolysis was demonstrable in mice given maximum sublethal doses of BL.

Cl. welchii antitoxin, which was allowed to act on BL at 37° for 30 min. before injection, though saving the life of BL-intoxicated mice, did not immunize completely. The protected animals, like the unprotected, became ill within 1–2 hr. of injection; but they recovered at the third hour, whereas the unprotected animals grew progressively worse until death. It is possible that this early illness was due to substances other than the lecithinase.

Haemolysis by BL

The haemolytic tests reported in our last paper (Miles & Miles, 1947) were extended to stronger preparations of BL. Table 3 records the HD 50 for red cells of various animal species, and the value of the lecithinase 'units' of various

Table 3. *Comparison of the* 50 % *haemolytic dose* (*HD* 50) *and lecithinase units, of various BL and WL preparations*

		Lecithinase preparation				
Red cells tested		M 10*g* BL 18 (mg.)	A 250*c* BL 17 (mg.)	M 58*e* BL 19 (mg.)	WL W'pool (mg.)	WL 290 (ml.)
HD 50	Human	> 20*	> 20*	> 20†	—	—
	Horse	> 20	> 20	> 20	—	—
	Sheep	> 20	> 20	> 20	—	—
	Rabbit	> 20†	> 20†	> 20‡	—	—
	Guinea-pig	> 20	> 20	> 20	—	—
	Mouse	3·75	8·0	0·625	0·625	0·00312
1 lecithinase unit (yolk extract)		0·10	0·04	0·05	0·3125	0·00156

— No test.
* Trace haemolysis at 20 mg./ml.
† 10 % haemolysis at 20 mg./ml.
‡ 10 % haemolysis at 5 mg./ml.
All recorded haemolysis neutralized by *Cl. welchii* α-antitoxin.

preparations of BL and WL. Our previous findings with 64 units BL/ml. are largely confirmed, but the use of preparations containing up to 500 units BL/ml. reveals minor activity against rabbit and human red cells. All the haemolytic activity of the BL preparations was neutralized by *welchii* α-antitoxin. Of the common laboratory animals, the mouse is the most convenient source of susceptible cells for haemolysin tests. The relative haemolytic inactivity of BL preparations in terms of 'lecithinase units', as compared with WL, is noticeable.

The efficacy of Cl. welchii *α-antitoxin against BL*

With the BL from strain M58*e*, it was possible to measure the serological relation between *Cl. bifermentans* lecithinase and the α-toxin of *Cl. welchii*, by titrating α-antitoxin, in mice, against approximately one LD50 of WL and BL. The mixtures of toxin and antitoxin were held for 30 min. at 37° before intravenous injection (Table 4). Though 13·5 mg. BL was about LD50, the dose

Table 4. *Protective power of* Cl. welchii *α-antitoxin in mice receiving approximately one LD50 of BL (M58e; BL20) and of WL (Welchpool)*

Antitoxin given (i.u.)	No. of mice dying/group given		Minimum protective dose of α-antitoxin (i.u.)
	13·5 mg. BL	0·15 mg. WL	
26·4	0/10	—	—
13·2	0/10	—	13·2
6·6	5/10	—	—
3·3	3/10	—	—
1·6	5/10	—	—
0·100	—	0/10	—
0·050	—	0/10	—
0·025	—	0/10	0·025
0·0125	—	3/10	—
0·000	4/10	9/10	—

given of WL W'pool proved to be about LD90; the protective dose of antitoxin will therefore be larger than that required for an LD50. The ratio of the minimal protective doses, measured on a twofold dilution scale is 528:1 (13·2:0·025). That is, compared with homologous toxin more than 500 times the amount of α-antitoxin is required for neutralization of the heterologous toxin.

Comparison of the lecithinase, haemolytic and toxic activities of BL and WL

Table 5 summarizes the results; the actual amounts in (*a*) are converted in (*b*) to percentages of the phosphate-liberating activity. The striking differences between BL and WL are the relatively high activity of BL in the 18 hr. yolk-extract titration, and the relatively low toxicity of BL. The high yolk-extract titres confirm our suggestion that BL is more stable than WL in mixtures with yolk extract. When yolk-extract titres are read at 1 hr. instead of 18 hr. the titre of BL is comparatively much lower. Of greater importance is the ratio of the LD50's in the two lecithinases. Averaging the results with WL290 and W'pool, the ratio is 670:72, or 9·3:1. That is, in terms of *in vitro* lecithinase activity at pH 7·5, BL is about one-ninth as toxic as WL.

The three preparations from different strains, though resembling one another more than they resemble WL, nevertheless differ significantly. The BL of A 250*c* and M 10*g* has less than half the toxicity for mice exhibited by BL M 58*e*, and a fourth to a sixth of its haemolytic activity. Assuming that 50 mg. is the LD 25 and LD 0 of M 10*g* and A 250*c* respectively (Table 2), and that the slope of the dose-mortality curves for all three preparations is the same, we may estimate the LD 50 of the two BL's very approximately as 170 and 200 mg. On

Table 5. *Comparison of the lecithinase, haemolytic and toxic potencies of preparations of BL and WL; (a) by weight or volume, and (b) in percentages of the amount liberating 50 μg. acid-soluble* P *in standard conditions*

	Strain	Preparation	DL 50 P (crude lecithin)	WL or BL unit (yolk ext.)	HD 50 (mouse cells)	LD 50 (mice)
(*a*)	A 250*c*	BL 17 (mg.)	4·63	0·04	8·00	> 50 (*c.* 200)
	M 10*g*	BL 18 (mg.)	3·13	0·10	3·75	> 50 (*c.* 170)
	M 58*e*	BL 20 (mg.)	2·24	0·05	0·625	15·01
	Welchpool	WL (mg.)	0·203	0·3125	0·625	0·15
	290	WL (ml.)	0·00143	0·00156	0·00312	0·00122
(*b*)	A 250*c*	BL 17	100	0·9	173	> 1080 (*c.* 4300)
	M 10*g*	BL 18	100	3·2	120	> 1562 (*c.* 5400)
	M 58*e*	BL 20	100	2·2	28	670
	Welchpool	WL	100	153·9	315	59
	290	WL	100	109·1	218	85

this basis A 250*c* and M 10*g* are between one-sixth and one-seventh as toxic as M 58*e*; i.e. toxicity roughly parallels haemolytic activity. From the corresponding values for toxicity in part (*b*) of the table, BL 17 and 18 were 60 and 75 times less toxic than WL, in terms of *in vitro* lecithinase activity. BL from M 10*g* and A 250*c* was prohibitively weak for further investigation of toxicity, but not for haemolysin tests. The varying ratio between DL 50 P and the haemolytic-toxic activity in the three preparations suggests that toxin and lecithinase are not the same. Accordingly, the amounts of a strong α-antitoxin neutralizing one HD 100 of BL 17, 18 and 20 were measured, and on one occasion proved to be respectively 30·5, 20·6 and 2·6 i.u. and on another, 29·0, 24·0 and 2·9 i.u. These figures, though not in the strict ratio of the haemolytic activities in terms of DL 50 P (173, 120, 28; Table 5*a*) are sufficiently close, within the limits of error of the tests, to suggest that the α-antitoxin which neutralizes lecithinase activity (Miles & Miles, 1947) also neutralizes the haemolysin in constant proportion. This result was not due to chance parallelism between α-antitoxin and a distinct antihaemolysin in the serum used; for when a number of α-antitoxic sera varying in α-antitoxin content between 3300 and 70 units/ml. were titrated against the haemolysin of BL 20 (M 58*e*), the neutralizing values were 2·6, 4·2, 3·8, 5·0, 9·0, 5·5, 4·9 and 3·5 units. With one exception (9·0) the values are sufficiently close to show that the α-antitoxin is the antihaemolysin. Another serum, containing 0·2 unit of α-antitoxin/ml. and large amounts of antibody to the β and θ lysins of *Cl. welchii*, had no antihaemolytic activity.

DISCUSSION

In our previous paper we suggested that the properties of the *bifermentans* lecithinase threw some doubt on the assumption that lecithinase activity *per se* was sufficient to account for the lethal and necrotic powers of the α-toxin of *Cl. welchii*, particularly because active BL was non-toxic compared with WL; and we concluded that the speculation could be tested fully only when we had a BL whose phosphate-liberating power was equivalent to that of at least 10 M.L.D. of *Cl. welchii* α-toxin. This we have achieved with BL 20; up to 50 mg. could be given to mice, which died only because of the *bifermentans* lecithinase effect, specifically neutralized by α-antitoxin. Fifty mg. contained the phosphate-liberating equivalent (Table 5) of $50/2{\cdot}24 = 22{\cdot}3$ DL 50 P of W'pool *welchii* toxin, and one DL 50 P of this toxin (0·203 mg., Table 5) was *c.* 1 M.L.D. (i.e. 1 LD 100, Table 2).

With this preparation of BL it is clear that, compared with WL, BL has about one-ninth the mouse-toxicity of WL, when lecithinase activity is defined in terms of acid-soluble P liberated under standard conditions. It is, of course, impossible to decide how far these conditions approximate to those in animal tissues that contain a substrate for the enzymes. All we have been able to do is to establish comparable temperature, pH, and a not too divergent tonicity. Our DL 50 P, like Macfarlane & Knight's lecithinase 'unit', which it closely resembles, is a highly artificial measure; and in view of the heterogeneity of the dose-response slopes in the titration of WL and BL by this means, comparisons of DL 50 P must be suspect unless in any one pair of comparisons the slopes are proved to be homogeneous. Nevertheless, a ninefold difference and, in the case of BL from two other strains, M 10*g* and A 250*c*, a much greater than ninefold difference in toxicity are probably significant. The differences, which were constant for several batches from each strain, between toxicities of BL from M 58*e* on the one hand, and the substantially non-toxic BL's from A 250*c* and M 10*g* on the other, also destroy any simple relation between lecithinase activity and toxicity for any one 'species' of lecithinase.

We suggested (1947) that differences between BL and WL might reside either in a readiness of absorption to certain tissue structures that is independent of lecithinase activity, or in the presence in the body of activators to one but not to the other. It now appears that similar notions apply to BL from different strains of *Cl. bifermentans*. In view of the impurity of our BL preparations, we cannot exclude synergic or inhibitory substances as the cause of the difference. Nevertheless, the constancy of this difference, exhibited by several batches of BL from each of the three strains, suggests that it is a property of the lecithinase itself. That is, there appear to be interstrain variations in some quality of the lecithinase, besides the power to hydrolyse lecithin, that determines toxicity. (This conclusion depends, of course, on the validity of our assumption that in BL preparations there is only one antigen—the lecithinase—capable of reacting with antibodies in *Cl. welchii* α-antitoxins.) It is, moreover, noteworthy that the varying susceptibility of the mouse to the lethal effect of enzymically equipotent doses of BL from the three strains studied, is paralleled

by the *in vitro* susceptibility of mouse red cells to haemolysis. Though haemolysis may not be the determining factor in toxicity, the red cell at least reflects the susceptibility of those other mouse tissues, whose poisoning determines the death of the animal.

The analysis of BL preparation yields no evidence that the serologically related *welchii* lecithinase is distinct from the α-toxin. On the other hand, though large amounts of BL are specifically toxic, it is clear that lecithinase activity *in vitro* and toxicity are not consistently related. The reasons for the comparative non-toxicity of BL are obscure, but it provides an explanation, additional to the fact that very little BL is formed by *Cl. bifermentans* in vigorously growing culture, for the lack of pathogenic power in the average strain of *Cl. bifermentans*. Chu (1949) records that the lecithinase of *Bacillus cereus* has one-quarter to one-eighth the toxicity of WL. His *cereus* lecithinases, though immunologically distinct from WL, were in other respects more closely related to WL than BL appears to be.

The importance of the antigenic relationship between BL and WL must not, however, be overrated. We have now established the reciprocity of the relation, in that WL antilecithinase neutralizes the mouse-toxicity of BL, and vice versa; and that it requires 500 times more α-antitoxin to neutralize one LD 50 of BL than one LD 50 of WL. But the chief value of the relationship has been in providing a reagent, *Cl. welchii* α-antitoxin, which, it was justifiable to assume, was monospecific with regard to BL. The significance of antigenic similarity of the two enzymes must await the establishment of more facts.

Finally, it is perhaps worth noting that our negative evidence for the identity of toxic and lecithinase activity in *Cl. welchii* α-toxin, and the positive evidence of Chu and ourselves that other lecithinases are toxic, does not in our opinion add much to the speculation that all bacterial exotoxins will prove to be enzymes when the relevant substrates are found. So far we have the unique fact that one naturally and obviously effective bacterial toxin appears to be an enzyme; and the other unrelated fact that certain enzymes, including some isolated from bacterial cultures, have proved to be toxic in concentrated preparations.

We are indebted to Dr W. M. Perry for the statistical analyses, and to Dr T. S. Work for the chromatographic analysis of the lecithinase hydrolysates.

REFERENCES

Bard, R. C. & McClung, L. S. (1948). Biochemical properties of the toxins of *Cl. novyi* and *Cl. haemolyticum*. *J. Bact.* **56**, 665.

Belfanti, S., Contardi, A. & Ercoli, A. (1936). Lecithinasen. *Ergebn. Enzymforsch.* **5**, 213.

Chu, H. P. (1949). The lecithinase of *Bacillus cereus* and its comparison with *Clostridium welchii* α-toxin. *J. gen. Microbiol.* **3**, 255.

Contardi, A. & Ercoli, A. (1933*a*). Sopra alcune scissioni enzimatiche della lisolecitina e della lecitina. Nuovi isomeri della lecitine. *Gazz. chim. ital.* **63**, 37.

Contardi, A. & Ercoli, A. (1933*b*). Über die enzymatische Spaltung der lecithine und Lysocithine. *Biochem. Z.* **261**, 275.

FISHER, R. A. (1948). *Statistical methods for research workers*, 10th ed. Edinburgh: Oliver and Boyd.

GADDUM, J. H. (1933). Methods of biological assay depending on a quantal response. *Spec. Rep. Ser. med. res. Coun., Lond.*, no. 183.

KING, E. J. (1947). *Micro-analysis in medical biochemistry*. London: J. and A. Churchill.

MACFARLANE, M. G. & KNIGHT, B. C. J. G. (1941). The biochemistry of bacterial toxins. I. The lecithinase activity of *Cl. welchii* toxins. *Biochem. J.* **35**, 884.

MILES, E. M. & MILES, A. A. (1947). The lecithinase of *Clostridium bifermentans* and its relation to the α-toxin of *Clostridium welchii*. *J. gen. Microbiol.* **1**, 385.

OETTINGEN, W. F. VON & BOWMAN, R. O. (1933). The physico-chemical properties of some new choline derivatives in relation to their chemical constitution and pharmacological action. *J. Pharmacol.* **48**, 336.

PALITZSCH, S. (1915). Ueber die Anwendung von Borax und Borsäurelösungen bei der colorimetrischen Messung der Wasserstoffion konzentration des Meerwassers. *Biochem. Z.* **70**, 333.

STAMP, LORD (1947). The preservation of bacteria by drying. *J. gen. Microbiol.* **1**, 251.

TREVAN, J. (1927). The error of determination of toxicity. *Proc. Roy. Soc.* B, **101**, 483.

(*Received* 22 *March* 1949)

A Note on the Globular Forms of *Vibrio cholerae*

By the late P. BRUCE WHITE
National Institute for Medical Research, Hampstead, London

SUMMARY: The cultivation of *Vibrio cholerae* in media containing sublethal concentrations of penicillin apparently favours the selection of two morphological forms: motile spherical masses containing large numbers of apparently nuclear elements, and fantastically branched forms. On penicillin-free media both forms yield the original comma forms.

Globular forms of *Vibrio cholerae* appear in stock cultures in the usual laboratory media, though not in 24 hr. cultures of strains freshly isolated from man. They may be produced in large quantities by adding about a quarter of the growth on a 24 hr. nutrient agar slope culture of *V. cholerae* to a solution of penicillin containing 25–100 u./ml. Upon incubation, mass growth takes place. The number of viable individuals does not increase, but each vibrio becomes a sphere, which may be up to 8–10 μ in diameter. The spheres are flagellated and motile, and remain so for several days in penicillin-vibrio mixtures held at 10°. Prolonged incubation of the mixtures produces larger (Pl. 1, figs. 1, 2) spheres up to 12 μ in diameter, which lose motility and staining properties, become 'vacuolated' and may bulge in subsidiary masses from the periphery. It is highly improbable that these spherical forms owe their motility to any form of torque, and it can only be concluded that the stainable flagella on their surfaces are indeed the cause of their locomotion.

By the differential congo-red methylene-blue method (White, 1947) the larger spheres commonly stain blue, and the remainder take up the methylene-blue only when they are dead. After extraction of the spheres to remove nucleic acids, staining with methylene-blue reveals large numbers of granules, which may represent the unit organisms in the vibrionic mass. The spheres have serological properties similar to the parent culture, whether these are Inaba or Ogawa in type.

The penicillin is neither lethal nor particularly injurious and appears simply to suppress directional growth and the usual mode of division, so that unoriented cell growth occurs, without increase in the number of viable individuals in the culture.

Under optimum conditions extraordinary starfish-like forms occur, with tapering branches; many of these may be motile. On staining, the broader part of the branches is seen to consist of double or multiple chains of nuclear elements; the finer branches are formed by simple vibrios, and from these the culture may regenerate, either in its original form, or, if seeded on to fresh penicillin-agar, in the spherical form.

I have considered the globular form as a step in the direction of the vesicular forms that occur in the streptobacilli, but have found no good evidence that this is the case. Another feature of general interest attaches to the branching forms derived from the multinuclear cells that occur both naturally and under

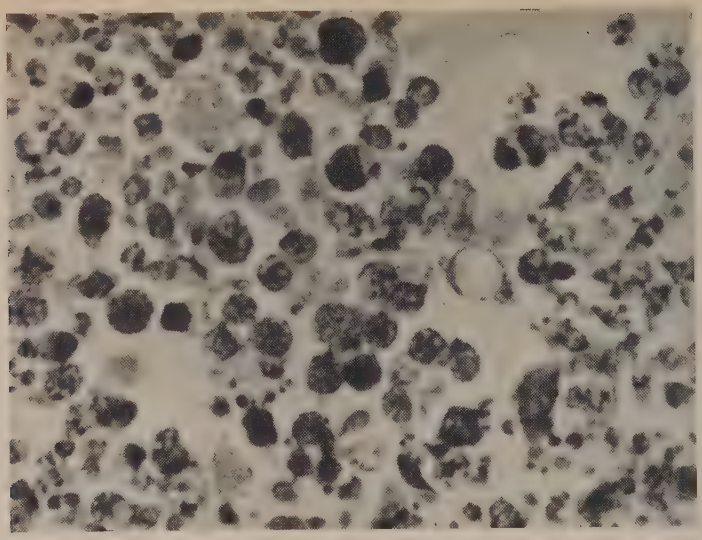


Fig. 1

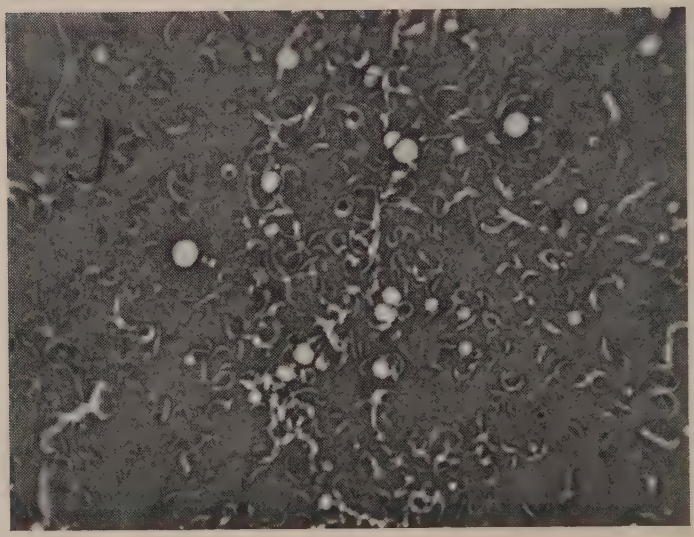


Fig. 2

P. Bruce White—Globular forms of *V. cholerae*. Plate 1

the influence of penicillin. It is possible that the development of multinuclearity in this way may have been a stage in the evolution of the multinucleate and branched species of bacteria.

REFERENCE

White, P. B. (1947). A method for combined positive and negative staining of bacteria. *J. Path. Bact.* 59, 334.

EXPLANATION OF PLATE

Fig. 1. Globular forms of *V. cholerae*, stained by methylene-blue. × 1250.

Fig. 2. Vibrionic, branched and globular forms of *V. cholerae*. Unstained preparation with a background of nigrosin. × 1250.

(*Received* 7 *April* 1949)

Chemical Factors in the Germination of Spore-bearing Aerobes: Observations on the Influence of Species, Strain and Conditions of Growth

By G. M. HILLS
Microbiological Research Department, Porton, near Salisbury, Wiltshire

SUMMARY: Some chemical requirements for stimulating the germination of spores of a few species of the genus *Bacillus* were studied by means of viable counts.

Of the three compounds alanine, tyrosine and adenosine, L-alanine was present in all cases where highly significant stimulation was observed; the need for the other two compounds ranged from ability to dispense completely with both of them to a distinct need for the two together to produce a maximum effect.

Generally, the effects were greater with spores grown in a chemically defined amino-acid medium aerated by shaking than with those grown on the surface of a casein-hydrolysate agar.

In the single case examined, that of a laboratory strain of *B. subtilis*, the effect of L-alanine was strongly inhibited D-alanine at a molar ratio of 1:30.

Previous papers (Hills, 1949*a*, *b*) have shown that the germination of spores of a virulent strain of *Bacillus anthracis* was stimulated by L-alanine, L-tyrosine and adenosine. Germination was strongly inhibited by D-alanine, and this inhibition was abolished by increasing the concentration of the L-isomer. After the discovery that only L-alanine was essential for rapid germination of the spores of a laboratory strain *B. subtilis*, a systematic examination of a number of species of the genus *Bacillus* was undertaken in order to see how far the chemical requirements for germination were common to the genus and how far they could be correlated with classification within the genus. The survey was not completed, but the data now available may be of interest to other workers studying the germination of spores.

METHODS

Strains used. Most of the work was done with a strain of *B. subtilis* isolated in this department. A virulent strain of *B. anthracis* supplied by Dr R. L. Vollum was that used previously by Hills (1949*a*, *b*). An avirulent strain of the same species, 34 F 2, was supplied by the Veterinary Laboratory of the Ministry of Agriculture, Weybridge. Other organisms were from the National Collection of Type Cultures (see Table 5).

Production of spore suspensions. Work with *B. subtilis* (laboratory strain) was carried out with a stock suspension which had been grown for 48 hr. on CCY agar (Gladstone & Fildes, 1940), heated 90 min. at 60° at a count of about 10^{10} spores/ml. and washed thrice with distilled water at a count of 2×10^9/ml.

For comparative work with other organisms, since it was considered that germination might be dependent on the method of cultivation, two different conditions of growth were used with each strain: (*a*) surface growth on CCY agar; (*b*) submerged growth, aerated by shaking, in the chemically defined

medium (AA) of Gladstone (1939) but with L-alanine in place of DL-alanine (Hills, 1949*b*) and supplemented with $1{\cdot}25 \times 10^{-6}$ M adenosine (Hills, 1949*a*), 5×10^{-4} M-Ca^{++}, 6×10^{-3} M-$NaHCO_3$ and $1{\cdot}4 \times 10^{-5}$ M-Mn^{++} (Brewer, McCullough, Mills, Roessler, Herbst & Howe, 1946) and 10^{-7} M aneurin (Hagan, O'Kane & Young, 1943). The metals were held in solution by M/100 citrate.

For optimal sporulation of some strains at 37°, growth on medium (*a*) was continued for 12 days. The organisms were then suspended in glass-distilled water, collected by centrifuging and washed. In medium (*b*) optimum sporulation was observed at 5 days. The organisms were then centrifuged out and washed. These times for optimum production of free spores were determined by microscopic examination, but viable counts of both types of suspension showed that the proportion of vegetative forms sensitive to heat (15 min. at 60°) was insignificant. These suspensions were used without heating to avoid the possibility, frequently reported (e.g. Robbins, Kavanagh & Kavanagh, 1942; Evans & Curran, 1943) of facilitating germination by heat treatment.

Experimental procedure. The media in which germination was studied were inoculated with about 10^4 spores/ml. and incubated at 35°. Counts of organisms which then remained viable after 15 min. at 60° (spore, or *S*-counts) were determined by the surface plate technique of Miles & Misra (1938). In selected experiments counts made without heating at 60° ($S + V$ counts, where V is the count of the vegetative form) showed little fall as a result of incubation, but in suitable media comparison with the *S*-counts showed that a high proportion of the spores had become sensitive to heat. Since these had remained viable during incubation they must be regarded as having germinated. In certain media, however, which were inadequate for growth though adequate for the process of sensitization to heat, loss of viability of the heat-sensitive forms was observed even at 35° on prolonged incubation. Nevertheless, in spite of difficulties due to death of germinated forms in media inadequate for growth, increased sensitivity to heat, as determined by viable count, is the best criterion of germination, since any method based solely on morphological changes cannot establish the most important feature of the process, namely, that changes from the characteristics of the spore state have not involved loss of viability. Further evidence in support of this view has recently been given by Wynne & Foster (1948). Technical details have been given elsewhere (Hills, 1949*a*), the only modifications being in the conditions of incubation of the plates according to the needs of the various organisms for the production of discrete colonies, uniformly 1–2 mm. in diameter.

Statistical treatment of data. The numbers of colonies on replicate plates conformed to a Poisson series, so that in assessing the significance of the differences between counts, according to standard methods (Fisher, 1946), the variance of any count could be taken equal to the count itself.

Materials. These were as used previously (Hills, 1949*a*, *b*). Throughout, molarities refer to a single enantiomorph; the total concentration of a DL-mixture is thus twice that given.

RESULTS

Experiments with Bacillus subtilis

Selection of medium. Fig. 1 shows the changes in S-count in a variety of media during 6 hr. incubation. These media fell into three groups:

(*a*) Disappearance of 50 % or more spores in the first 2 hr. occurred only in the amino-acid medium (A), in CCY, and in media containing tryptic digest of meat (TM and TM + PB). The initial fall in the number of spores with CCY occurred 1 hr. later than in the other media, but the subsequent decrease in S-count was rapid, followed later by an increased count due to resporulation.

(*b*) In the second group, which gave significant but less than 50 % germination in the first 2 hr. and little further increase, the gelatin-tyrosine medium (GT), developed for the assay of yeast fractions in the germination of *B. anthracis*, was not significantly different from phosphate buffer (B), and was not improved by yeast extract (GTY).

(*c*) The replacement of the amino-acids of medium (A) by ammonium salts inhibited germination since the medium (NH_4) allowed less change in spore count than the phosphate buffer (B) which it contained.

All the changes observed were presumably due to germination, since considerable growth occurred in those cases with the most rapid decrease in S-count. This was shown by very high $S + V$ counts at the end of the experiment, using the same sized sample as for the S-counts but without heating at 60°. Some growth also occurred in the gelatine-tyrosine medium with yeast (GTY), giving a twofold increase in the count of an unheated dilution, but in the remaining three media, B, GT and NH_4, this count was either lower than or not significantly different from the initial count.

Table 1. *Effect of incubation in amino-acid media on spores of a strain of* Bacillus subtilis

All media were buffered with M/30 phosphate, pH 7·3.
S = spore count; $S + V$ = spore and vegetative form count.

			Medium			
				5 mM-L-alanine 5 mM-DL-tyrosine	17 amino-acids as in Fig. 1A	
		Buffer				
15 μM-adenosine		0	0	+	0	+
Viable count (in 0·05 ml.)	Initial	855	810	793	850	848
	At 2 hr. S	688	47	36	572	491
	At 2 hr. $S + V$	772	227	118	713	699

Since measurable rates of germination were observed in the 'complete' amino-acid medium, it was compared with a simpler amino-acid medium (Table 1) containing L-alanine and tyrosine which, with the addition of adenosine, had been found adequate for *B. anthracis*. Fig. 1 shows that even with the 'complete' medium there was only 50 and 70 % germination in 1 and 2 hr. respectively; the latter experimental period therefore was chosen. The simpler medium, however, caused a much more rapid fall in S-count, but in

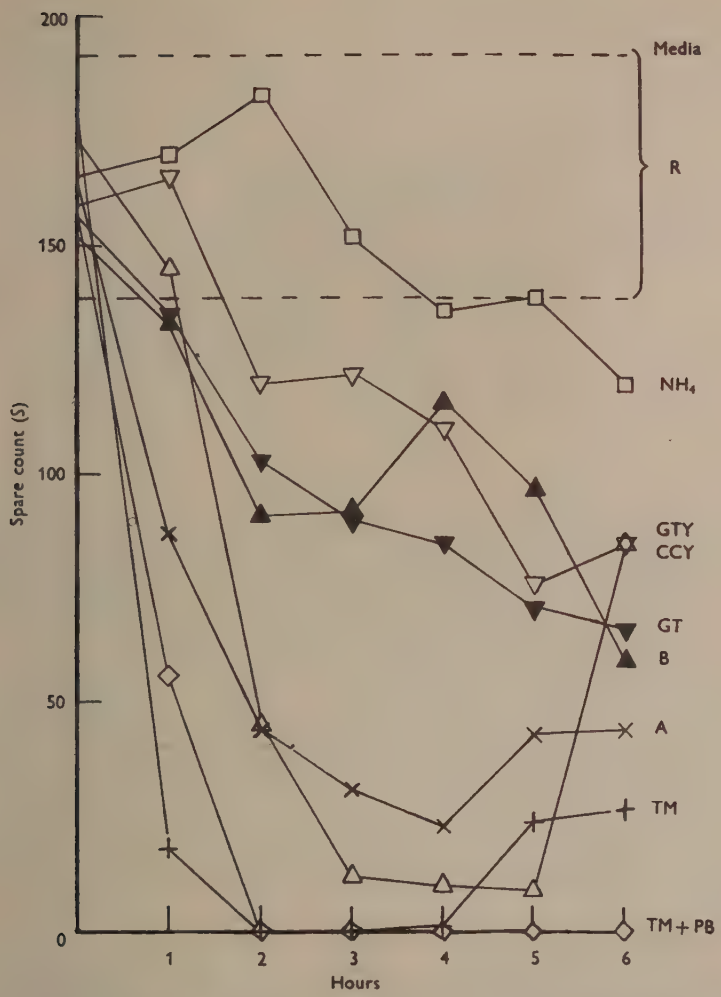


Fig. 1. The effect of incubation (35°) in various media on S-counts with a strain of *B. subtilis*. The sample for each count was 0·2 ml. of a 1/10 dilution in M/30-phosphate buffer pH 7·3. The count of spores and vegetative cells together ($S+V$) at the end of the experiment is indicated with the key to the media used. R = range of counts not significantly different from the mean initial count ($P=0{\cdot}95$).

Media (with final $S+V$ count): A, 17 amino-acids (Hills, 1949*b*, Table 1, with Na citrate but no threonine), × (∞); B, M/30 phosphate buffer pH 7·3, ▲ (105); CCY, acid + tryptic hydrolysates of casein + yeast extract (Gladstone & Fildes, 1940), △ (∞); GT, gelatin hydrolysate + tyrosine (Hills, 1949*a*), ▼ (162); GTY, GT + yeast extract, ▽ (361); NH_4, ammonium salts at concentrations used by Fildes (1938) in place of amino-acids of A, □ (140); TM, tryptic digest of beef + (∞); TM + PB, TM + Fildes's peptic sheep's blood ◇ (∞).

2 hr. the $S+V$ count also fell considerably, so that it was not possible to ascribe the decreased S-count to germination, rather than to a sporicidal effect. The only significant effect of adenosine in this medium was a greater decrease in $S+V$ count. In a subsequent experiment, limited to 1 hr. incubation (Table 2), L-alanine was the only component essential for the decrease in

Table 2. *Effect of alanine and tyrosine on germination of a strain of* Bacillus subtilis

The basal medium was M/30 phosphate pH 7·3, and the amino-acids were added at a concentration 5 mM.

		Additions to basal medium			
		None	L-alanine	DL-tyrosine	L-alanine DL-tyrosine
		Viable count (per 0·05 ml.)			
Time (hr.)	Count				
0	$S+V$	238	233	256	214
	S	216	215	252	214
1	$S+V$	196	212	212	190
	S	192	51	229	68

S-count, but it had no effect on the total number of viable organisms, which, although showing a 10–20 % decrease as a result of incubation, showed the same decrease when in phosphate buffer alone. Since 75 % germination was observed in 1 hr., the experimental period was shortened to 30 min. in subsequent experiments and good germination still took place.

Alanine was not replaceable by lactate or pyruvate, even at 5–10 times the alanine concentration and in the presence of an equivalent of NH_4^+, though none of these compounds significantly inhibited the decrease in S-count caused by alanine. Thus in 28 mM phosphate buffer of pH 7·3, buffer with 5 mM-NH_4Cl, 5 mM pyruvate, pyruvate + NH_4Cl, 2·5 mM-DL-lactate, DL-lactate + NH_4Cl, the count, initially in the range 669–717, decreased slightly to 627–662 after incubation for 30 min. With 0·5 mM-L-alanine in addition to any of the above substrates or combinations of substrates the final count was in the range 123–162.

Inhibition by D-*alanine.* As with *B. anthracis* the decrease in S-count caused by L-alanine was inhibited by D-alanine, a molecular ratio of 0·03 D- to L-alanine allowing about twice as many spores to survive as with L-alanine alone at 0·5–5 mM (Table 3). Controls with the brucine and strychnine used in the resolution of DL-alanine showed a small inhibition of germination at 20 μg./ml. in the presence of 500 μM-L-alanine, but even 1·35 μg./ml. D-alanine (15 μM) caused an inhibition about three times as great. This effect of D-alanine therefore cannot be due to traces of these alkaloids as impurities.

Experiments with other organisms

Table 4 shows the effects on the S-counts after incubation in phosphate buffer pH 7·3 with the addition of L-alanine, DL-tyrosine and adenosine singly and in all possible combinations. The organisms used were four strains of

B. anthracis and a strain each of *B. cereus*, *B. megatherium* and *B. subtilis*. Two spore suspensions of each organism were used, grown respectively on the surface of CCY agar (Gladstone & Fildes, 1940) and in an amino-acid fluid medium aerated by shaking (see Methods).

Table 3. *Inhibition of germination of a strain of* Bacillus subtilis *by* D-*alanine*

The basal medium was M/30-phosphate, pH 7·3. The initial *S*-counts, both with and without D- or L-alanine, formed a homogeneous population of range 515–611 ($P = 0{\cdot}95$).

Conc. of L-alanine (μM)	Conc. of D-alanine (μM) 0	15	50	150
	S-count at 30 min.			
0	529	528	612	622
500	157	341	467	594
1500	129	160	235	388
5000	79	85	118	162

No experiments were carried out with either *B. mesentericus* or *B. mycoides*. Two strains of the former (N.C.T.C. nos. 2589 and 6223) although sporing satisfactorily on CCY agar, failed to grow in the defined medium; and two strains of *mycoides* (N.C.T.C. nos. 926 and 2602) did not spore satisfactorily on either medium and were also unsuitable for study by the technique used, on account of the difficulty of getting discrete colonies for counting.

Except with *B. cereus* those conditions which produced a significant decrease in *S*-count gave a greater decrease with spores grown in the amino-acid medium than with those grown on CCY. Although this difference was, in general, quantitative rather than qualitative, it led in certain cases to qualitative differences due to limitations of the counting technique in detecting small changes (5–18 % depending on the total number of colonies for both counts of which the significance of the difference was required). Thus with *B. anthracis*, N.C.T.C. 5444, 79 % of spores grown on the amino-acid medium were susceptible to the action of alanine and tyrosine together but the corresponding figure of 15 % for spores of this strain grown on CCY agar was not significantly different from the control. On the other hand, with *B. anthracis*, N.C.T.C. 109, both figures, 88 and 25 % respectively, were significant.

The simplest requirements for spore germination were shown by *B. cereus* for which alanine alone was effective, though there was some improvement with tyrosine or adenosine, and these two together, without alanine, gave a small but significant effect. All four strains of *B. anthracis*, whether virulent or avirulent, had similar requirements. Alanine, tyrosine and adenosine together were significantly better than any single component or combination of two components. Again, alanine seemed to be of greatest importance since only two cases in 24 showed a significant effect ($P = 0{\cdot}95$) in its absence, and in one of these cases the decrease in *S*-count was so small that the probability of such a difference in the same direction arising by chance was nearly 1 in 50. With both the Weybridge and Vollum strains, the effect of alanine in diminishing

Table 4. *The effect of adenosine, alanine and tyrosine on germination of some species and strains of the genus* Bacillus

The media for cultivation of spores were CCY (Gladstone & Fildes, 1940) or medium AA with 18 amino-acids (defined in text). The basal medium for examination of germination was M/30-phosphate at pH 7·3. Duration of incubation 30 min. Materials added were adenosine (Ad), 16 μM; L-alanine (Al), 500 μM; DL-tyrosine (Ty), 500 μM. Significant changes in spore count have been italicized, i.e. with a decrease from the control exceeding twice its standard error.

Bacillus species ...	*anthracis*								*cereus*		*megatherium*		*subtilis*	
Strain ...	Vollum		Weybridge		N.C.T.C. 109		N.C.T.C. 5444		N.C.T.C. 2599		N.C.T.C. 6347		N.C.T.C. 3610	
Spores cultivated on	CCY	AA	CCY	AA	CCY	AA	CCY	AA	CCY	AA	CCY	AA	CCY	AA
No. plates for each treatment	3	3	3	3	3	3	3	3	6	6	3	3	3	6
Final *S*-count in basal medium (total colonies)	466	499	600	490	380	620	243	510	597	3205	570	642	641	1485
	Effect of additions on final *S*-count (%)													
Additions to basal medium														
Ad	+ 6	*−21*	− 2	+ 8	− 5	− 6	0	+ 1	*−16*	− 3	+ 1	− 7	+ 1	− 5
Al	− 2	− 8	0	+ 6	− 1	*−25*	−15	*−20*	*−73*	*−58*	+ 1	*−22*	− 1	*−15*
Ty	+ 1	− 1	+ 3	+ 4	− 8	0	− 8	− 2	− 3	0	+ 1	− 2	+ 4	− 5
Ad+Al	*−14*	*−37*	− 4	*−50*	*−24*	*−48*	−12	*−32*	*−94*	*−84*	*−27*	*−22*	+ 1	*−19*
Al+Ty	*−13*	*−45*	+ 2	−10	*−25*	*−88*	−15	*−79*	*−78*	*−77*	+ 1	*−22*	+ 2	− 9
Ty+Ad	−12	−11	0	+ 7	− 9	*−12*	− 5	−10	*−23*	*−10*	− 5	− 6	+ 2	− 7
Ad+Al+Ty	*−58*	*−91*	*−89*	*−93*	*−89*	*−93*	*−49*	*−88*	*−96*	*−93*	*−35*	*−23*	− 6	*−13*

the S-count in the presence of tyrosine and adenosine was subsequently shown to be due to germination. Under conditions in which the S-count fell to 10–35 % of its original value in the presence of alanine, the $S+V$ count in the presence or absence of alanine and the S-count in the absence of alanine fell only to 85–95 % of the original value of the $S+V$ count, of which the original S-count was 95–99 % ($P=0{\cdot}95$). Germination of *B. megatherium* and *B. subtilis* was slower so that large decreases in S-count were not observed during the period of the experiment. Significant decreases were observed only in the presence of alanine, but these were small and were not affected, in the case of cultures grown in the amino-acid medium, by the other components either singly or together. During the period of the experiment, S-counts gave no evidence for an effect of any of the materials on the germination in phosphate buffer of *B. subtilis* spores grown on the surface of CCY agar. A significant fall in the count of spores of *B. megatherium*, grown on CCY agar, was produced only in the presence of alanine and adenosine together, with or without tyrosine.

DISCUSSION

This work was begun in order to seek a correlation between chemical requirements and mode of germination, such as the differences observed by Lamanna (1940), namely, absorption of the spore-coat in large-celled species and shedding of the spore-coat in small-celled species. No generalization is possible since too few species have been studied, and strain variations in *B. anthracis* suggest the need for the study of a number of strains of each species. The variations obtained with the strains of *B. anthracis*, however, were quantitative rather than qualitative. All strains were more exacting in the need for tyrosine and adenosine than the single strain of *B. cereus* studied. The smaller variations in requirements within the species were not correlated with virulence; thus the virulent 'Vollum' was less exacting than the avirulent 'Weybridge', but more so than the avirulent N.C.T.C. no. 5444.

The failure of *B. mycoides* (N.C.T.C. nos. 926 and 2602) to spore under the conditions used was unfortunate, since the work of Knaysi (1945) indicates requirements for germination in this species different from those of the organisms studied here. Using a microscopic technique, Knaysi found that a nitrogen source was unnecessary, buffered glucose being adequate for 30 % germination in 5 hr.; KNO_3 facilitated the conversion of germinating forms to true vegetative cells. His conditions, however, must have been far from optimal, since Robinow (1942), studying cytological changes during germination, has shown that in broth marked changes occur during the first 5 or 10 min. of incubation and that cell division occurred in about 1 hr. These rates are comparable with those observed with the organisms studied here. Moreover, Knaysi & Baker (1947) have explained germination in nitrogen-free media by exhaustion of cellular ribonucleic acid, so that some nitrogen source is essential to give normal, as distinct from deficient vegetative cells.

Owing to the use of different strains and experimental conditions, the results with *B. subtilis* are not directly comparable with those of Keilin & Hartree

(1947), who found that N.C.T.C. no. 89 needed a stable dialysable factor from yeast extract, peptone or tryptic digest of casein. The present findings are that L-alanine in phosphate buffer was a sufficient stimulus for a laboratory strain, but N.C.T.C. no. 3610 did not respond, in a short incubation period, either to this or to the alanine + tyrosine + adenosine medium which was adequate for *B. anthracis*, and in which adenosine replaced a yeast extract (Hills, 1949*a*) having similar properties to that used by Keilin & Hartree.

The changes in *S*-count have not in every case been proved to be due to germination, but this was the case with two strains of *B. anthracis* and with a laboratory strain of *B. subtilis*. With *B. subtilis* loss of viability was observed in the absence of heat treatment only after prolonged incubation of suspensions containing germinated forms in media inadequate for growth. It is probable, therefore, that germination was the cause of the decrease in *S*-count in experiments of short duration with other species.

L-alanine was a constituent of the medium in 40 cases out of 42 where the decrease in *S*-count exceeded that which would have been expected by chance in 1 case in 50. The importance of L-alanine is emphasized by the inhibition of its effect by D-alanine at a molar ratio of 1:30 in the case of *B. anthracis* (Hills, 1949*b*) and by a laboratory strain of *B. subtilis* which required only L-alanine to stimulate 80 % germination in 1 hr. at 35°. The specificity of the effects of the isomers of alanine on the germination of bacterial spores is at present unique in bacterial chemistry in the low ratio to L-alanine at which D-alanine inhibits. The inhibition of *Lactobacillus arabinosus* by D-leucine and D-valine (Fling & Fox, 1945) or by glycine (Kobayashi, Fling & Fox, 1948) and the inhibition of *Escherichia coli* by these amino-acids or by D-alanine (Kobayashi, Fling & Fox, 1948) required high concentrations approaching 0·1 M. The view that the large side-chain of valine or leucine is essential for steric hindrance cannot now be regarded of general biological application, and its bearing on the mode of action of peptide antibiotics containing D-amino-acids (Fox, Fling & Bollenbach, 1944) is now in doubt (cf. Work, 1948).

I have much pleasure in thanking Dr D. W. Henderson and Dr D. D. Woods for careful criticism of this paper and Dr H. N. Rydon for samples of D- and L-alanine and synthetic DL-tyrosine. The experimental work was carried out with the technical assistance of Cpl. W. Bailey, R.A.M.C., while the author was a member of the Scientific Staff, Medical Research Council. Permission to publish has been granted by the Chief Scientist, Ministry of Supply.

REFERENCES

Brewer, C. R., McCullough, W. G., Mills, R. C., Roessler, W. G., Herbst, E. J. & Howe, A. F. (1946). Studies on the nutritional requirements of *Bacillus anthracis*. *Arch. Biochem.* **10**, 65.

Evans, F. R. & Curran, H. R. (1943). The accelerating effect of sub-lethal heat on spore germination in mesophilic aerobic bacteria. *J. Bact.* **46**, 513.

Fildes, P. (1938). The growth of *Proteus* on ammonium lactate plus nicotinic acid. *Brit. J. exp. Path.* **19**, 239.

Fisher, R. A. (1946). *Statistical methods for research workers*, 10th ed. Edinburgh: Oliver and Boyd.

FLING, M. & FOX, S. W. (1945). Antipodal specificity in the inhibition of *Lactobacillus arabinosus* by amino-acids. *J. biol. Chem.* **160**, 329.

FOX, S. W., FLING, M. & BOLLENBACH, G. N. (1944). Inhibition of bacterial growth by D-leucine. *J. biol. Chem.* **155**, 465.

GLADSTONE, G. P. (1939). Interrelationships between amino-acids in the nutrition of *B. anthracis*. *Brit. J. exp. Path.* **20**, 189.

GLADSTONE, G. P. & FILDES, P. (1940). A simple culture medium for general use without meat extract or peptone. *Brit. J. exp. Path.* **21**, 161.

HAGAN, W. A., O'KANE, D. J. & YOUNG, G. A. (1943). Private communication.

HILLS, G. M. (1949*a*). Chemical factors in the germination of spore-bearing aerobes. The effect of yeast extract on the germination of *Bacillus anthracis* and its replacement by adenosine. *Biochem. J.* **45**, 353.

HILLS, G. M. (1949*b*). Chemical factors in the germination of spore-bearing aerobes. The effect of amino-acids in the germination of *Bacillus anthracis* including observations on the specificity of some stereoisomers. *Biochem. J.* **45**, 363.

KEILIN, D. & HARTREE, F. (1947). Comparative study of the spores and vegetative forms of *Bacillus subtilis*. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **12**, 115.

KNAYSI, G. (1945). Investigation of the existence and nature of reserve material in the endospore of a strain of *Bacillus mycoides* by an indirect method. *J. Bact.* **49**, 617.

KNAYSI, G. & BAKER, R. F. (1947). Demonstration, with the electron microscope, of a nucleus in *Bacillus mycoides* grown in a nitrogen-free medium. *J. Bact.* **53**, 539.

KOBAYASHI, Y., FLING, M. & FOX, S. W. (1948). Antipodal specificity in the inhibition of growth of *Escherichia coli* by amino-acids. *J. biol. Chem.* **174**, 391.

LAMANNA, C. (1940). The taxonomy of the genus *Bacillus*. 1. Modes of spore germination. *J. Bact.* **40**, 347.

MILES, A. A. & MISRA, S. S. (1938). The estimation of the bactericidal power of blood. *J. Hyg., Camb.*, **38**, 732.

ROBBINS, W. J., KAVANAGH, V. W. & KAVANAGH, F. (1942). Growth substances and dormancy of spores of *Phycomyces*. *Bot. Gaz.* **104**, 224.

ROBINOW, C. F. (1942). A study of the nuclear apparatus of bacteria. *Proc. Roy. Soc.* B, **130**, 299.

WORK, T. S. (1948). D- and L-amino-acids in antibiotics. *Biochem. Soc. Symp.* **1**, 61.

WYNNE, E. S. & FOSTER, J. W. (1948). Physiological studies in spore germination with special reference to *Clostridium botulinum*. 1. Development of a quantitative method. *J. Bact.* **55**, 61.

(*Received* 29 *April* 1949)

The Non-toxicity of *Bacillus anthracis* Cell Material

By H. K. KING and JEAN H. STEIN
The Bacteriology Department, University of Edinburgh

SUMMARY: Suspensions of *Bacillus anthracis*, grown under various conditions, and disintegrated by shaking with minute glass particles, were not toxic to mice. The organisms were grown on both simple and complex media, aerobically and anaerobically, with and without extra carbon dioxide, for periods of from 6 hr. to 10 days.

The highly pathogenic *Bacillus anthracis* has always presented a puzzle to pathologists since no toxin has ever been demonstrated (for references, see Cromartie, Bloom & Watson, 1947). Toxin production may require special conditions of culture not yet determined (cf. Gladstone, 1946, 1948); also, the preparation from a sporing organism of sterile extracts for animal experiments presents difficulties. The method of rapidly disintegrating bacteria by shaking with minute glass beads (Curran & Evans, 1942; King & Alexander, 1948) has now been used for attempting the demonstration of an endotoxin in *B. anthracis*, since spores are as readily destroyed as vegetative organisms. Though complete sterility is not always easily attained, the vast majority of cells in a suspension are killed by a few hours' shaking, while as far as is known labile cell constituents, e.g. enzymes, are not damaged (Gale, 1946).

METHODS

Organisms. Two strains of *B. anthracis* were used: strain 'D' from our laboratory stock, and N.C.T.C. no. 5180. The two organisms were passaged through mice three times and the approximate minimum lethal dose (M.L.D.) was then found to be 2000 organisms for each strain.

Media. The following media were used; the abbreviations are those used in Table 1: (*a*) nutrient agar (N.A.); (*b*) blood agar (B.A.), nutrient agar with the addition of 5 % of sterile horse blood; (*c*) peptone water (P.W.), 1 % peptone; (*d*) the horseflesh digest (H.F.D.) of Mackie & McCartney (1948); (*e*) blood broth (B.B.), nutrient broth with addition of 5 % sterile horse blood; (*f*) serum broth (S.B.), nutrient broth with addition of 20 % horse serum. The blood media were incubated before use to test their sterility.

Conditions of culture. 'Aerobic' signifies culture in a shallow layer of medium with free access of air; 'semi-aerobic', in a closed 500 ml. bottle half-filled with medium; 'anaerobic', growth in a McIntosh & Fildes anaerobic jar. In some cases a gas-phase enriched with carbon dioxide was used. The incubation temperature was usually 37° but sometimes 22°.

Technique. The medium was inoculated and incubated for the time and under the conditions shown in Table 1. The organisms were then harvested—by washing off with saline from solid media, or by centrifugation from liquid media. The harvested cells were washed twice on the centrifuge with saline. With blood broth, the washing was performed with distilled water to lyse the

erythrocytes. The organisms were finally suspended in a suitable volume of saline and the approximate concentration of the suspensions determined by means of Brown's opacity tubes.

The organisms were disintegrated as follows. In most cases 40 ml. of suspension were transferred to a 4 oz. screw-cap flat medicine-type bottle containing 40 g. of grade 12 'ballotini'; i.e. glass balls with an average diameter of 0·13 mm. (Messrs Chance Bros., Smethwick, England). The bottle was placed lengthwise in the shaking machine and shaken horizontally at 500 strokes/min. with a 2½ in. stroke until sterility was achieved or, in a few cases, until the viable count had fallen to a sufficiently low value for the number of live organisms remaining in the material injected to be only a small fraction of the M.L.D. Smaller vessels were used for small volumes. The technique was essentially that of King & Alexander (1948), but a higher rate of shaking permitted readier attainment of sterility. All vessels and reagents were sterile and aseptic precautions were observed throughout. A viable count was performed on the disintegrated material, which was regarded as sterile if no live organisms were found in 0·1 ml.

A suitable quantity of the disintegrated suspension was injected into mice, usually 0·5 ml. intraperitoneally, but sometimes 0·2 ml. subcutaneously or intravenously. The dose was usually adjusted to contain the equivalent of about 500×10^6 organisms. When very dense suspensions were shaken it was difficult to attain sterility or even a sufficient decrease in the viable count. In Exp. 30 where $70{,}000 \times 10^6$ organisms in 1 ml. saline were given, the disintegrate was centrifuged and the sterile supernatant used for the test. Usually three mice were used for each test, and the result was considered negative if the animals survived 7 days. Cultures were made from the spleen and heart blood of any animals dying within this period.

In all experiments with liquid media, the culture fluid was examined for exotoxins, though their presence was most unlikely. The supernatant was filtered through a '5/3' sintered glass filter and the sterile filtrate tested for toxin by injecting 0·5 ml. intraperitoneally into each of three mice.

RESULTS

The results are set out in Table 1. In no case were the disintegrated organisms or the culture filtrates lethal, with the exception of a few cases in which *B. anthracis* was recovered from the heart or spleen, or in which other pathogenic organisms, e.g. *Salmonella*, were found. Since these experiments yielded no evidence of toxin formation, we considered the possibility of toxin being produced *in vivo*. The spleens of anthrax-infected mice were selected as the source of material; these usually contain large numbers of the bacilli at death. The mice were infected and either allowed to die, or killed when moribund; this reduced the risk of a labile toxin being destroyed by the autolytic enzymes liberated at death. The spleens of two or three mice were removed and transferred aseptically to a sterile 25 ml. screw-capped bottle containing 5 ml. water and 5 g. ballotini and sterilized by shaking for 10 hr. The resulting material was freeze-dried, each spleen yielding about 10 mg. dry material. There was

Table 1. *Preparations of disintegrated* Bacillus anthracis *which proved to be non-toxic when injected into mice*

Exp. no.	Strain of organisms	Medium	Temp. of incubation	Duration of incubation	Gaseous phase	Duration of shaking (hr.)	Equivalent total no. of dead organisms injected (millions)	Volume injected (ml.)	Route
1	D	N.A.	37°	24 hr.	Aerobic	24	20	0·2	s.c.
2	D	B.A.	37°	12 hr.	Aerobic	4	150	0·2	s c.
3	D	B.A.	37°	24 hr.	Aerobic, 30% CO_2	24	300	0·2	s.c.
4	5180	B.A.	22°	24 hr.	Aerobic	6	400	0·2	s.c.
5	5180	P.W.	37°	24 hr.	Aerobic	4½	370	0·5	i.p.
6	5180	P.W.	22°	3 days	Aerobic	6	900	0·5	i.p.
7	5180	P.W.	22°	3 days	Aerobic, 30% CO_2	3	370	0·5	i.p.
8	5180	P.W.	37°	24 hr.	Anaerobic	4½	370	0·5	i.p.
9	5180	P.W.	37°	48 hr.	Anaerobic, 10% CO_2	1	200	0·5	i.p.
10	D	B.B.	37°	20 hr.	Aerobic	24	300	0·2	s.c.
11	D	B.B.	37°	20 hr.	Anaerobic	24	300	0·2	s.c.
12	5180	B.B.	37°	18 hr.	Aerobic	6	370	0·5	i.p.
13	5180	B.B.	37°	10 days	Aerobic	18	600	0·2	i.p.
14	5180	B.B.	22°	3 days	Aerobic	6	500	0·5	i.p.
15	5180	B.B. pH 8	37°	24 hr.	Aerobic	4	370	0·5	i.p.
16	5180	B.B. pH 6	37°	24 hr.	Aerobic	4	370	0·5	i.p.
17	5180	B.B.	37°	6 hr.	Semi-aerobic	6	370	0·5	i.p.
18	5180	B.B.	37°	24 hr.	Semi-aerobic	6	370	0·5	i.p.
19	5180	B.B.	37°	24 hr.	Anaerobic, 10% CO_2	6	370	0·5	i.p.
20	5180	S.B.	37°	18 hr.	Aerobic	6	900	0·5	i.p.
21							500	0·2	i.v.
22	5180	S.B.	37°	18 hr.	Anaerobic	5	370	0·5	i.p.
23	5180	S.B.	37°	20 hr.	Aerobic	29	1,800	0·5	i.p.
24							700	0·2	i.v.
25	5180	H.F.D.	37°	5 days	Aerobic	6	500	0·5	i.p.
26							200	0·2	i.v.
27	5180	B.B.	37°	16 hr.	Aerobic	20	2,000	0·2	i.v.
28	D	N.A.	37°	14 hr.	Aerobic	12	6,000	0·2	i.v.
29							30,000	1·0	i.v.
30	D	B.A.	37°	20 hr.	Aerobic	60	70,000	1·0	i.p.

Abbreviations. Media, see p. 48. Route of administration; s.c. subcutaneous; i.p. intraperitoneal; i.v. intravenous.

no means of estimating the number of bacteria present or even the proportion of the dry weight which was bacterial material. No deaths occurred when mice were injected with the following doses: from spleens of mice which died, 0·3 mg. in 0·2 ml. saline, intravenous, or 3·9 mg. in 0·5 ml. saline, intraperitoneal; from spleens of mice killed when moribund, 1·4 mg. in 0·2 ml. saline, intravenous, or 3·6 mg. in 0·5 ml. saline, intraperitoneal.

DISCUSSION

Violent shaking with minute glass beads, as used here, is believed to break open bacterial cells and to liberate the contents with relatively little damage to labile proteins. The period of shaking was considerably longer than is usually employed, for example, for the extraction of enzymes, where the aim is the disintegration of the majority of cells without necessarily sterilizing the culture. Even so we did not achieve sterility in every case, but always succeeded in so reducing the viable count that the volumes injected contained far less than the M.L.D. of the live organism. The prolonged shaking also involved risk of destruction of proteins by autolytic enzymes liberated from the cells. This point required special consideration in view of Gladstone's (1948) findings that the extracellular antigen of anthrax is readily destroyed by the organism's own proteolytic enzymes. Shaking was carried out at room temperature (15° in most of our experiments) when enzyme action would be relatively slow. We consider that only a substance of quite exceptional lability would be destroyed under our conditions, and the validity of this method for treating endotoxins was demonstrated in a control experiment with *Vibrio cholerae* in which the M.L.D. of disintegrated organisms was shown to be the same as for those killed by mild heat.

Bearing in mind that a toxin might be produced only under certain special conditions, we used a wide range of cultural conditions, attempting in some cases to simulate the natural internal environment of the host. We used only moderate doses of the killed organisms to avoid confusing the issue with non-specific protein toxicity. Dense suspensions proved difficult to sterilize; nevertheless, our mice resisted doses of $30{,}000 \times 10^6$ organisms. The cell material of the anthrax bacillus thus appears to possess a surprisingly low toxicity, and our results are in accord with the pessimistic view of Cromartie *et al.* (1947) that 'Our knowledge of anthrax in this respect (its pathogenic mode of action) has not been added to since the turn of the century'.

We wish to thank Prof. I. J. Mackie, C.B.E., for his interest in this work, which was performed during the tenure by one of us (H. K. K.) of the Lewis Cameron Teaching Fellowship of the University of Edinburgh.

REFERENCES

Cromartie, W. J., Bloom, W. L. & Watson, D. W. (1947). Studies on infection with *Bacillus anthracis*, I. *J. infect. Dis.* **80**, 1.

Curran, H. R. & Evans, F. R. (1942). Killing of bacterial spores in fluids by agitation with small inert particles. *J. Bact.* **43**, 125.

Gale, E. F. (1946). The bacterial amino-acid decarboxylases. *Advances Enzymol.* **6**, 1.

Gladstone, G. P. (1946). Immunity to anthrax: protective antigen present in cell-free filtrates. *Brit. J. exp. Path.* **27**, 394.

Gladstone, G. P. (1948). Immunity to anthrax: production of the cell-free protective antigen in cellophane sacs. *Brit. J. exp. Path.* **29**, 379.

King, H. K. & Alexander, H. (1948). The mechanical destruction of bacteria. *J. gen. Microbiol.* **2**, 315.

Mackie, T. J. & McCartney, J. E. (1948). *Handbook of practical bacteriology*, 8th ed. p. 147. Edinburgh: E. and S. Livingstone.

(*Received* 30 *April* 1949)

Interpreting Relationships between the Concentrations of Plant Viruses and Numbers of Local Lesions

By A. KLECZKOWSKI
Rothamsted Experimental Station, Harpenden, Hertfordshire

SUMMARY: The effect of dilution on numbers of local lesions produced by plant viruses was tested graphically and statistically for compatibility with contrasting hypotheses. Experimental results are incompatible with the hypothesis that lesions are produced because of chance encounters between single virus particles and susceptible regions of a uniform type, and that variations in response to similar inoculation between different leaves or plants occur solely because of variations in numbers of such regions. The results are compatible with the hypothesis that individual susceptible regions vary in susceptibility so that different doses of virus are needed to produce a lesion (the variations being such that the logarithms of minimal effective doses are normally distributed). The second hypothesis is, therefore, more probably correct, but the first can be fitted to experimental results by introducing various auxiliary assumptions, such as the existence of qualitatively different virus particles and susceptible regions. Conclusive evidence is unlikely to come from mathematical treatment of existing results, but only from a new experimental approach.

There are two theories concerning the effect of concentration of virus inoculum on the production of a local lesion. According to one theory a local symptom develops as a result of the presence of at least one virus particle in the inoculum which has been suitably introduced into susceptible host tissue. This is analogous to the presence or absence of bacterial growth depending on the presence or absence of at least one viable bacterium in the volume of fluid inoculated upon a suitable medium. This theory does not allow for any variation in susceptibility between different susceptible regions of the host. Differences between hosts would arise wholly because of variation in numbers of susceptible regions.

The other theory assumes that susceptible regions vary in susceptibility so that the minimum virus concentration necessary to initiate local infection varies from one region to another.

Youden, Beale & Guthrie (1935) and Bald (1937*a*, *b*, *c*) dealing mainly with tobacco mosaic virus, and Parker (1938) who worked with vaccinia virus, concluded that the numbers of lesions obtained with different dilutions of inocula approximately fit the equation derived from the Poisson series on the assumption that a local lesion is caused by the introduction into a 'susceptible region' of at least one virus particle. On the other hand, Bryan & Beard (1940) concluded that their results with papilloma virus and Parker's results with vaccinia virus are better fitted by the assumption that regional susceptibility varies in such a way that the logarithm of minimal effective concentration is normally distributed. This is analogous to the susceptibility of animals to chemical reagents, such as drugs or poisons, which cause 'quantal' responses. Logarithms of minimal effective doses are usually normally distributed (Gaddum, 1933; Bliss, 1935; Finney, 1947).

Lauffer & Price (1945) considered both theories and concluded that the theory of local infection, caused by single virus particles entering uniformly susceptible regions, is compatible with experimental data obtained with all viruses so far investigated, whereas data obtained with plant viruses are definitely incompatible with the theory of variation in regional susceptibility. Their conclusions were not substantiated by statistical tests, but were based on approximate graphical fitting (judged by inspection) of experimental data to the curves of the equations derived from the two theories. As they point out, the χ^2 test is inapplicable to results obtained with local lesions produced by plant viruses, although it can be, and has been, applied by Haldane (1939) to Parker's results with vaccinia virus, and by Bryan & Beard (1940) to their results with papilloma virus. With these animal viruses the test was applicable because known numbers of susceptible sites were inoculated with known volumes of the inoculum, whereas with local lesions produced on plants inoculated by rubbing their leaves with virus solutions, neither is known. Dilution-infection series obtained with plant viruses, however, can be tested statistically for compatibility with theoretical assumptions by comparing two estimates of the same variance, one obtained from analysis of variance of all data in a given experiment, and the other from deviations of experimental from theoretical values. This can be done by making use of the fact that suitably transformed local-lesion counts are approximately normally distributed with a variance independent of the mean (Kleczkowski, 1949).

In the work described below, dilution-infection series were obtained with plant viruses and tested for their compatibility with each of two hypotheses. It was undertaken for three reasons. First, Lauffer & Price's arguments for rejecting the hypothesis of host variation in regional susceptibility were considered inadequate. Secondly, it was considered that the published dilution-infection series do not sufficiently cover the ranges of high virus concentration. Thirdly, it was thought desirable to analyse the results of at least a few experiments statistically. Published data could not be used for this purpose because the method employed necessitates knowing the individual lesion counts for each replication, whereas only the total numbers of lesions for each virus concentration have been published.

EXPERIMENTAL

Most of the experiments were made with tobacco mosaic virus (TMV) on *Nicotiana glutinosa*. Purified preparations of the virus were used in most experiments, but sap from infected tobacco plants was used in some. A few experiments were made with purified preparations of tomato bushy stunt virus (BSV) on *N. glutinosa*, and with the Rothamsted culture of tobacco necrosis virus (TNV) using sap from infected tobacco plants as inocula and bean plants (*Phaseolus vulgaris*, var. Prince), in the two primary leaves stage, as the test plant.

The plants were inoculated by rubbing the leaf surfaces with the forefinger wet with inoculum. Each dilution of the inoculum was rubbed on a number of half-leaves distributed in such a way as to eliminate as many sources of

variation as possible. The experimental design shown in Table 1 was used when eight different virus concentrations were tested on *Nicotiana glutinosa*. Each plant was trimmed to six comparable leaves. Each treatment had twelve replications and occurred three times in each of the four blocks of two plants,

Table 1.

Leaf position ...	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.
I	1	4	5	8	2	5	6	1	3	6	7	2	4	7	8	3
II	2	3	6	7	3	4	7	8	4	5	8	1	5	6	1	2
III	3	2	7	6	4	3	8	7	5	4	1	8	6	5	2	1
IV	4	1	8	5	5	2	1	6	6	3	2	7	7	4	3	8
V	5	8	1	4	6	1	2	5	7	2	3	6	8	3	4	7
VI	6	7	2	3	7	8	3	4	8	1	4	5	1	2	5	6
Plant	*a*		*b*		*c*		*d*		*e*		*f*		*g*		*h*	
Block	*A*				*B*				*C*				*D*			

Table 2.

Leaf position ...	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.
I	1	2	3	4	3	1	7	5	1	4	3	2	5	1	2	6
II	6	5	8	7	2	4	6	8	8	5	6	7	7	3	4	8
Plant	*a*		*b*		*c*		*d*		*e*		*f*		*g*		*h*	
Block	*A*				*B*				*C*				*D*			

Leaf position ...	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.
I	1	6	5	2	7	1	2	8	1	8	5	4
II	8	3	4	7	4	6	5	3	7	2	3	6
Plant	*i*		*j*		*k*		*l*		*m*		*n*	
Block	*E*				*F*				*G*			

twice on each leaf position and six times each on the left (L.) and on the right (R.) half-leaf. When eight virus concentrations were tested on beans the experimental design shown in Table 2 was used. Only half of the whole design is shown. The other half is a repetition of the first but with the order L.–R. reversed. The two primary leaves of beans occupy the same position on the stem, giving one systematic variation fewer than with *N. glutinosa*. Each treatment had fourteen replications and occurred once in each of the fourteen blocks of two plants. Each treatment occurred with every other twice on one leaf, and four times on the same plant but on the opposite leaves.

RESULTS

Graphical fittings

In this section approximate graphical comparisons, judged by inspection, are made between experimental data and the curves of the equations based on the assumptions under test. In the next section the data of a few experiments are tested statistically for compatibility with the assumptions.

The curves of the equations:

$$Y = N\,(1 - e^{-ax}), \tag{1a}$$

$$Y = N\,\{1 - e^{-ax}\,(1 + ax)\}, \tag{1b}$$

$$Y = N\,\{1 - e^{-ax}\,(1 + ax + [a^2x^2/2\,!])\} \tag{1c}$$

and

$$Y = N\,\{1 - e^{-ax}\,(1 + ax + [a^2x^2/2\,!] + [a^3x^3/3\,!])\}, \tag{1d}$$

obtained from the Poisson series on the assumption that at least 1, 2, 3 and 4 virus particles, respectively, are necessary to produce a lesion in any of the N accessible susceptible regions, are shown in Fig. 1A.

Y is the expected number of lesions per half-leaf, N is the mean number of the 'susceptible regions' per half-leaf, x is the virus concentration (in g./l.) or the dilution of infective sap, and a is a constant. N and a are the parameters of the equations. The values of the parameters are unknown and have to be so adjusted for each equation to obtain the best possible fit to the experimental data.

Fig. 1B shows the curve of the equation

$$Y = \frac{N}{\lambda\sqrt{(2\pi)}} \int_{-\infty}^{t} \exp\left\{-\frac{1}{2}\left(\frac{t-\xi}{\lambda}\right)^2\right\} dt, \tag{2}$$

which corresponds to the assumption that 'susceptible regions' vary in susceptibility in such a way that logarithms of minimal virus concentrations, necessary to cause formation of a lesion, are normally distributed.

Y is the expected number of lesions per half-leaf, N is the mean number of 'susceptible regions' per half-leaf, $t = \log_{10} x$ (x = virus concentration in the inoculum or dilution of infective sap), $\xi = \log_{10} x_0$ (x_0 = virus concentration or dilution of infective sap when 50 % of the susceptible regions develop lesions) and λ is the standard deviation. This equation has three parameters, N, ξ and λ, the values of which are unknown and have to be adjusted to give the best possible fit to the experimental data.

Table 3 gives the results of fifteen dilution-infection series, and their graphical fittings to equations (1*a*) and (2) are shown in Figs. 2–5.

In Exps. nos. 1–12 the inocula were either sap from infected plants or purified virus preparations with the highest concentration of 1 g./l. or less. Figs. 2A–4A show that the results of some experiments, notably nos. 3, 5–9, 11 and 12, could be fitted satisfactorily to equation (1*a*). (The deviation in Exp. no. 11, Fig. 4A, of the point corresponding to the highest virus concentration from the curve can be explained by the inhibitory effect of the undiluted sap used as inoculum.) The results of Exp. nos. 1, 2, 4 and 10 could not be fitted to equation (1*a*), as the curve slopes too steeply. The points of Exp. no. 10 (Fig. 4A) could be fitted satisfactorily by disregarding the point corresponding to the highest concentration.

In Exps. nos. 13–15 the highest virus concentration was 20 g./l., and the results could not be fitted to equation (1*a*), the slope of the curve being too steep. The numbers of lesions were still increasing with increasing virus concentration up to a concentration of 20 g./l.

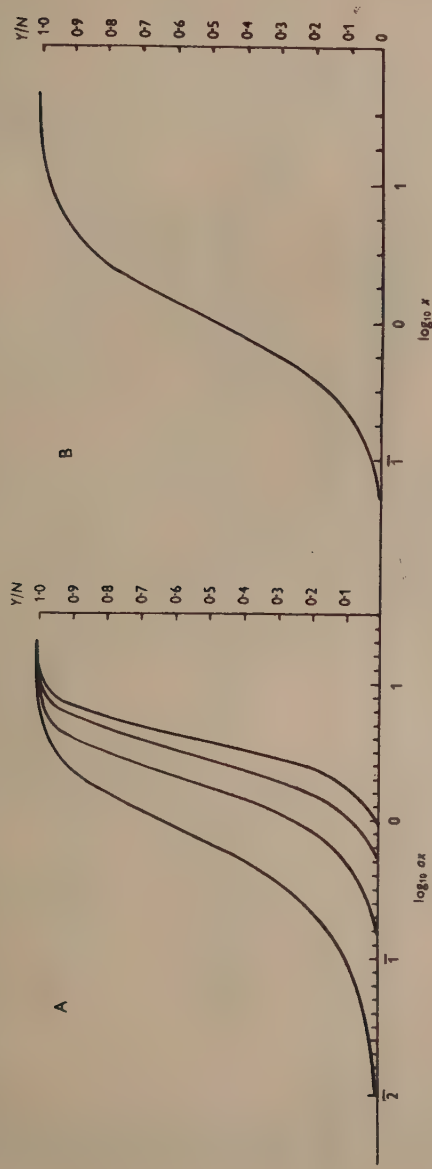


Fig. 1. A. The curves of equations (1 *a–d*). B. The curve of equation (2) when $\xi = 0$ and $\lambda = 0{\cdot}5$.

Several other dilution-infection series with the highest virus concentration at 20 g./l. were made, and the results depended on the susceptibility of the test

Table 3. *Numbers of lesions obtained by inoculating viruses at different concentrations*

Exp. no. 1 BSV	Exp. no. 2 TMV	Exp. no. 3 TMV	Exp. no. 4 TMV	Exp. no. 5 TMV
$c = 1{\cdot}0$ g./l.	$c = 0{\cdot}2$ g./l.	$c = 0{\cdot}2$ g./l.	$c =$ sap 1/2	$c = 1{\cdot}0$ g./l.
$f = 2$	$f = 2$	$f = 2$	$f = 3{\cdot}16$	$f = 3{\cdot}16$
$n = 24$	$n = 24$	$n = 24$	$n = 12$	$n = 12$
3491	3482	769	1525	2298
3344	2970	742	1288	2285
2829	2641	423	722	1650
2538	1674	282	551	989
1256	1377	138	362	511
1055	791	121	207	367
898	600	69	67	67
618	378	34	42	42
414	193	12		
231	81	18		
121	97	10		
41	42	8		

Exp. no. 6 TMV	Exp. no. 7 TMV	Exp. no. 8* TMV	Exp. no. 9 TMV	Exp. no. 10 TMV
$c =$ sap 1/5	$c =$ sap 1/5	$c = 0{\cdot}3$ g./l.	$c = 1{\cdot}0$ g./l.	$c = 1{\cdot}0$ g./l.
$f = 3{\cdot}16$	$f = 3{\cdot}16$	$f = 4$	$f = 3{\cdot}16$	$f = 3{\cdot}16$
$n = 12$	$n = 12$	$n = 12$	$n = 12$	$n = 12$
1216	1066	3453	1097	3848
1052	439	1589	910	2747
621	219	914	677	2603
253	166	270	396	2063
189	99	94	160	868
72	61	23	70	529
18	12	5	33	160
8	9	4	7	58

Exp. no. 11 TNV	Exp. no. 12 TNV	Exp. no. 13 TMV	Exp. no. 14 TMV	Exp. no. 15 TMV
$c =$ undil. sap	$c =$ undil. sap	$c = 20{\cdot}0$ g./l.	$c = 20{\cdot}0$ g./l.	$c = 20{\cdot}0$ g./l.
$f = 3{\cdot}16$	$f = 3{\cdot}16$	$f = 5$	$f = 5$	$f = 3{\cdot}16$
$n = 14$	$n = 14$	$n = 12$	$n = 12$	$n = 12$
1790	708	2906	1426	826
2055	632	2510	1162	808
1403	414	1626	653	590
921	208	1484	461	478
343	91	972	179	376
133	41	628	82	174
38	5	276	43	152
15	4	146	8	122
				30
				17

c = inoculum containing the highest virus concentration in a given experiment.
f = factor by which other inocula were consecutively diluted.
n = number of half-leaves on which given numbers of lesions were obtained.
* The leaves were sprinkled with 'Celite' (diatomaceous silica Filter-Aid, Johns-Manville) before inoculation in order to increase the numbers of lesions formed.

plants used. With less susceptible plants the results resembled those shown in Fig. 5. The leaves of more susceptible plants were covered with coalescing

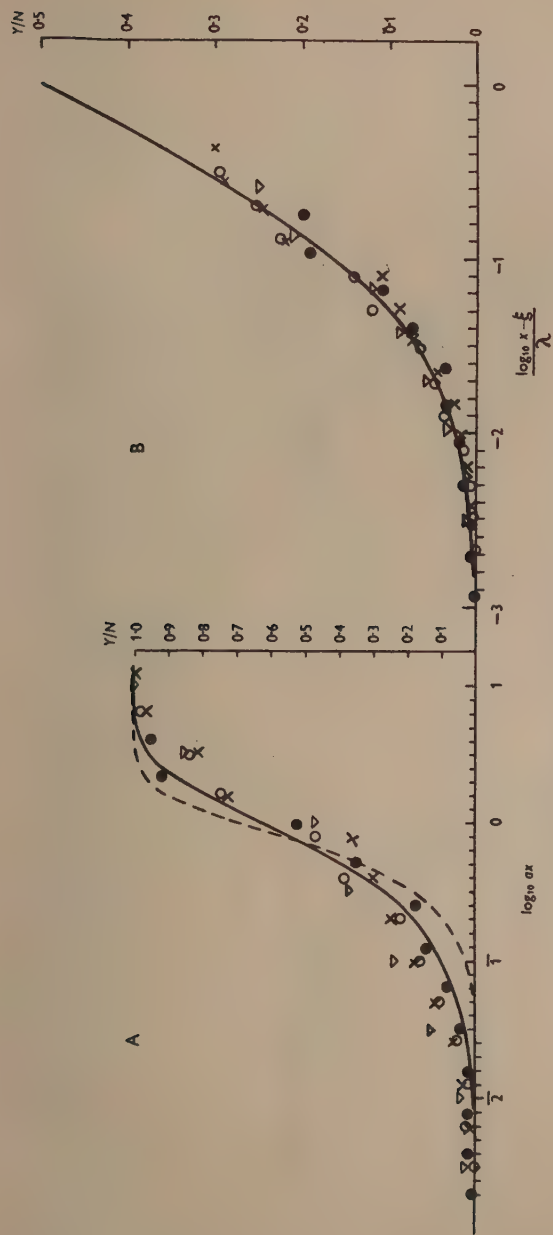


Fig. 2. Fitting the experimental results to the curve:

		(A) of equation (1 *a*)	(B) of equation (2)
× Exp. no. 1	(BSV)	$a = 12{\cdot}6$; $N = 146$	$\xi = +0{\cdot}57$; $\lambda = 1{\cdot}62$; $N = 485$
○ Exp. no. 2	(TMV)	$a = 31{\cdot}5$; $N = 148$	$\xi = +0{\cdot}05$; $\lambda = 1{\cdot}5$; $N = 493$
● Exp. no. 3	(TMV)	$a = 19{\cdot}9$; $N = 34$	$\xi = +0{\cdot}32$; $\lambda = 1{\cdot}36$; $N = 160$
▽ Exp. no. 4	(TMV)	$a = 20{\cdot}0$; $N = 127$	$\xi = +0{\cdot}8$; $\lambda = 1{\cdot}82$; $N = 508$

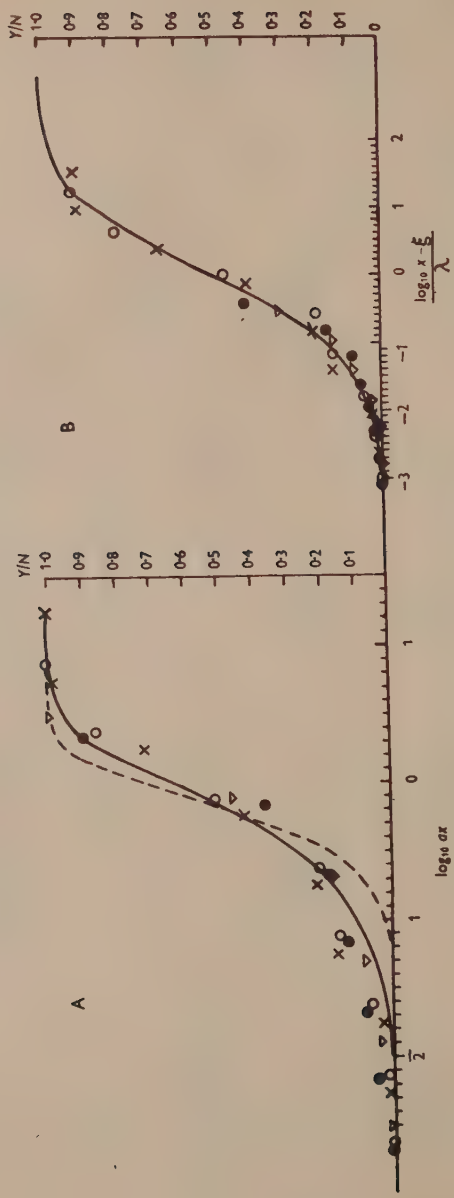


Fig. 3. Fitting the experimental results to the curve:

		(A) of equation (1*a*)	(B) of equation (2)
× Exp. no. 5	(TMV)	$a = 17{\cdot}8$; $N = 192$	$\xi = -1{\cdot}3$; $\lambda = 0{\cdot}83$; $N = 218$
○ Exp. no. 6	(TMV)	$a = 37{\cdot}5$; $N = 101$	$\xi = -1{\cdot}7$; $\lambda = 0{\cdot}83$; $N = 113$
● Exp. no. 7	(TMV)	$a = 11{\cdot}2$; $N = 89$	$\xi = -0{\cdot}1$; $\lambda = 1{\cdot}8$; $N = 213$
▽ Exp. no. 8	(TMV)	$a = 10{\cdot}5$; $N = 288$	$\xi = +0{\cdot}23$; $\lambda = 1{\cdot}33$; $N = 959$

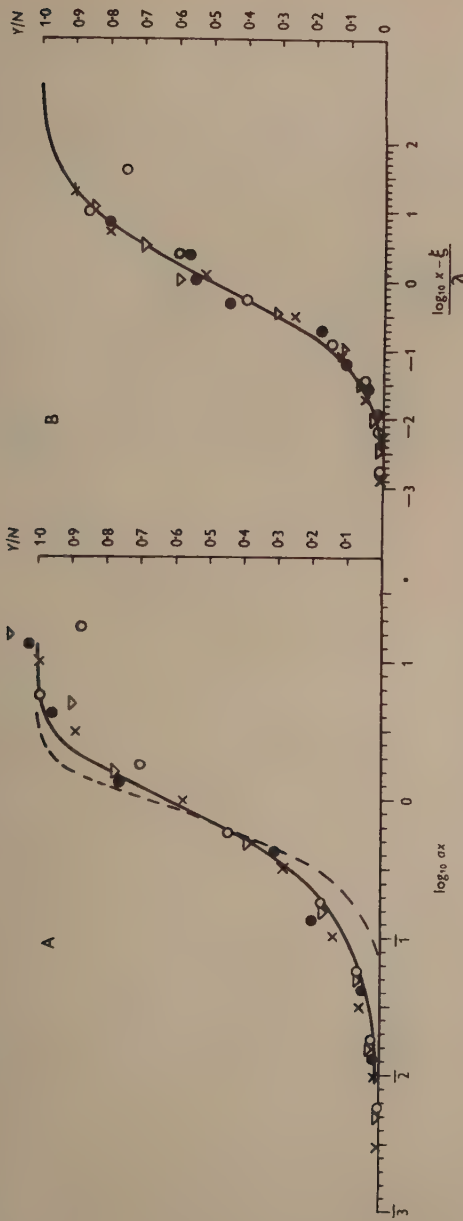


Fig. 4. Fitting the experimental results to the curve:

		(A) of equation (1*a*)	(B) of equation (2)
▽ Exp. no. 9	(TMV)	$a = 15{\cdot}85$; $N = 84$	$\xi = -1{\cdot}0$; $\lambda = 1{\cdot}0$; $N = 108$
● Exp. no. 10	(TMV)	$a = 42{\cdot}5$; $N = 222$	$\xi = -1{\cdot}06$; $\lambda = 1{\cdot}25$; $N = 401$
○ Exp. no. 11	(TNV)	$a = 17{\cdot}8$; $N = 147$	$\xi = -1{\cdot}3$; $\lambda = 0{\cdot}8$; $N = 170$
× Exp. no. 12	(TNV)	$a = 10{\cdot}0$; $N = 51$	$\xi = -1{\cdot}1$; $\lambda = 0{\cdot}83$; $N = 56$

lesions when the virus concentration was much less than 20 g./l. As these lesions were uncountable, no comparisons with any of the curves could be made.

The curve shown by broken lines in Figs. 2A–4A is that of equation (1*b*) based on the assumption that at least two virus particles are needed to cause a lesion. The curve is placed so that its 50 % point coincides with that of the curve of equation (1*a*). The slope of this curve is obviously too steep to fit any of the experimental data shown in Figs. 2–5. This also excludes the possibility

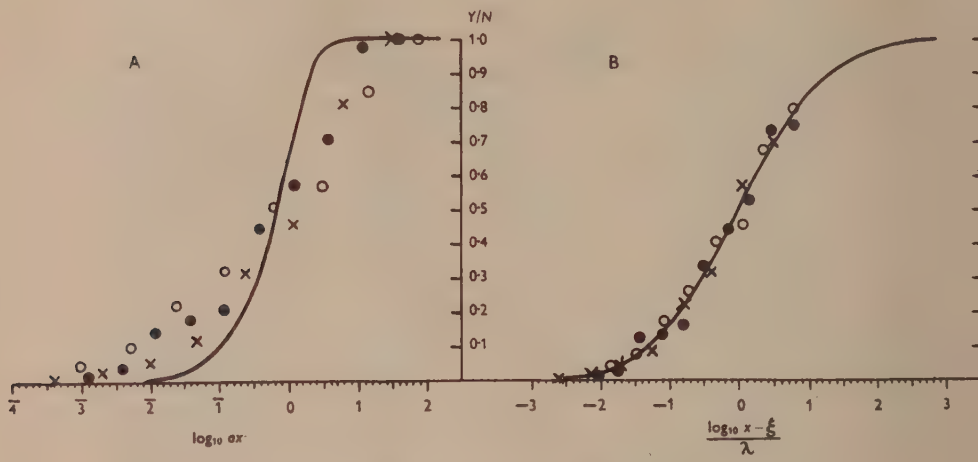


Fig. 5. Fitting the experimental results to the curve:

		(A) of equation (1*a*)	(B) of equation (2)
○ Exp. no. 13	(TMV)	$a=4{\cdot}0$; $N=242$	$\xi=-0{\cdot}17$; $\lambda=1{\cdot}84$; $N=280$
× Exp. no. 14	(TMV)	$a=1{\cdot}58$; $N=119$	$\xi=+0{\cdot}5$; $\lambda=1{\cdot}59$; $N=170$
● Exp. no. 15	(TMV)	$a=2{\cdot}0$; $N=69$	$\xi=+0{\cdot}05$; $\lambda=1{\cdot}56$; $N=92$

of fitting the curves of the equations for at least three, four or more particles, as their slopes are progressively steeper.

Figs. 2B–5B show that the results of all the experiments described here could be fitted approximately to equation (2), based on the assumption that the logarithm of the minimal effective virus concentration is normally distributed. The deviation of the point corresponding to the highest concentration of TNV in Exp. no. 11, Fig. 4B, from the curve can be explained, as previously remarked, by the inhibiting effect of undiluted sap used as inoculum.

Statistical analysis

A value c can be found such that the value $z=\log_{10}(y+c)$, where y is the number of lesions per half-leaf, is approximately normally distributed with a standard error independent of the mean (Kleczkowski, 1949). Statistical analyses of some of the dilution-infection series were made using this transformation. A preliminary value of c to be used for the results of a particular

experiment was estimated by using the regression equation of standard errors of the numbers of lesions per half-leaf for each virus dilution on the mean number for the same dilution. The value of c is equal to the distance (in the negative direction) from the origin to the point of intersection of the regression line with the axis of abscissae. This procedure was repeated using the transformed value $z = \log_{10}(y+c)$ instead of y for the estimation of standard errors for each virus dilution. If a regression line, almost parallel to the axis of the abscissae was obtained, the value of c was considered satisfactory; otherwise it was suitably readjusted.

When a hypothesis leading to any of the equations (1 *a–d*) or (2) is tested, the value $Y_i = \phi(x_i)$ given by the equation is assumed to be the theoretical arithmetic mean of numbers of lesions per half-leaf corresponding to a given virus concentration x_i, whereas the mean values of $z = \log_{10}(y+c)$ are logarithms of geometric means of the experimental values $(y+c)$. The theoretical arithmetic mean has, therefore, to be transformed into the logarithm of geometric mean, which can then be compared with the mean value of z.

If the logarithms of the population of values $(y+c)$ are normally distributed with the mean μ and the variance σ^2, the arithmetic mean Y plus the constant c is

$$Y + c = E(10^z) = \frac{1}{\sigma\sqrt{(2\pi)}} \int_{-\infty}^{\infty} 10^z \exp\left\{-\frac{1}{2\sigma^2}(z-\mu)^2\right\} dz$$
$$= 10^{\mu} e^{\frac{1}{2}(2\cdot 3\sigma)^2} = M_g\, e^{2\cdot 645\sigma^2},$$

where $M_g = 10^{\mu}$ is the theoretical geometric mean. Thus

$$\log_{10} M_g = \log_{10}(Y+c) - 1\cdot 149\sigma^2.$$

The value of σ^2 is unknown, but its estimate, s^2, is obtained from the analysis of variance of the experimental results transformed into $z = \log(y+c)$. Thus the theoretical value of $\log_{10} M_g$ will be approximately given by

$$Z = \log_{10}(Y+c) - 1\cdot 149 s^2, \tag{3}$$

so that $E(Z) = \log_{10} M_g$.

The parameters of the equations $Y = \phi(x)$ were adjusted by the method of least squares, i.e. by minimizing the value $\Sigma(\bar{z}_i - Z_i)^2$, where $\bar{z}_i$'s are the sample means of n values of z_i for each virus concentration x_i and Z_i's are the values of Z for the same virus concentration, obtained from equation (3). As all $\bar{z}_i$'s are assumed to have the same variance, this procedure is equivalent to the method of maximum likelihood.

If the assumptions on which the function $Y = \phi(x)$ is based are true, the value $\frac{n}{W}\Sigma(\bar{z}_i - Z_i)^2$ will give an estimate of the variance σ^2, based on W degrees of freedom, independently of that given by s^2, obtained from the analysis of variance. The number of the degrees of freedom, W, is equal to the number of treatments (i.e. different virus concentrations) minus the number of adjustable parameters in the equation $Y = \phi(x)$. Whether or not the two estimates of σ^2 are compatible is tested by the z-test or by the variance ratio test. When the ratio of the greater to the smaller estimate was found significant, the assumptions on which the tested function $Y = \phi(x)$ is based were considered incompatible with the experimental data.

Only treatments that gave a mean number of not less than five lesions per half-leaf could be analysed in this way, for if the means are smaller the transformation $z = \log_{10}(y+c)$ cannot be expected to make the variance independent of the mean (Kleczkowski, 1949).

The results of only three experiments (nos. 9, 10 and 13) were analysed because the analysis is too time-consuming. The three were chosen because each gave results of different type. All fitted approximately to the curve of equation (2). Only one (no. 9) fitted to the curve of the equation (1*a*). No. 13 obviously did not fit to it, and no. 10 could be fitted only if the point corresponding to the highest virus concentration was disregarded.

Table 4. *Analyses of variances*

Transformation→	Exp. no. 9 $z=\log_{10}(y+3)$			Exp. no. 10 $z=\log_{10}(y+20)$			Exp. no. 13 $z=\log_{10}(y+20)$		
	D.F.	Sums of squares	Mean squares (s^2)	D.F.	Sums of squares	Mean square (s^2)	D.F.	Sums of squares	Mean square (s^2)
Between treatments	5	9·9611		7	14·1082		7	8·9727	
Between blocks	3	0·6217		3	0·4708		3	2·0667	
Between leaf positions	—	— *		5	0·9951		5	1·0180	
Residual	63	2·9854	0·0474	80	2·9825	0·0373	80	2·2305	0·0286
Total	71	13·5682		95	18·5566		95	14·2879	

* The sum of squares was 0·1004 with 5 degrees of freedom. No precision would be gained by including this in the analysis of variance.

The analyses of variances for the three experiments are given in Table 4. All the experiments had eight treatments (virus concentrations) with twelve replications of each, and the experimental design was that shown above. In experiment no. 9 the two lowest virus concentrations gave mean numbers of less than five lesions per half-leaf so the results obtained with these were not used. All the data obtained in the two other experiments were used.

Tables 5–7 give the results of comparisons of the experimental values of $\bar{z}$ of Exp. nos. 9, 10 and 13 with computed values of Z, obtained from equation (3) by substitution values for Y obtained from the equations (1*a*, *b*) and (2), the parameters of which were adjusted by the method of least squares. The equation

$$Y = N_1(1-e^{-a_1x}) + N_2(1-e^{-a_2x}) \qquad (4)$$

was also tested with the results of Exp. no. 13, the meaning of which is discussed later.

Equation (1*a*) could be fitted satisfactorily to the results of Exp. 9 but not to those of Exp. 10. It could be fitted to no. 10 only if the result obtained with the highest virus concentration was disregarded. Equation (1*b*) does not fit the results of Exps. nos. 9 and 10, even if the result obtained in Exp. no. 10 with the highest virus concentration was disregarded. Fig. 5A shows that equations (1*a* and *b*) obviously do not fit the results of Exp. no. 13, so no statistical test of fit of these equations was made. Equation (2) can be fitted satisfactorily to the results of all three experiments. It is also shown that equation (4) can be fitted to the results of Exp. no. 13.

DISCUSSION

The fact that equation (1 *a*) did not fit the results of all the experiments must alone be considered sufficient for the conclusion that the assumptions from which it was derived is false. There are, however, other reasons for its rejection.

Table 5. *Comparisons of experimental with computed values for Exp. no.* 9

TMV conc. (g./l.)	$Y=\phi(x)\rightarrow$ $\bar{z}$	I (1 *a*) Z	II (1 *b*) Z	III (2) Z	Parameters I	Parameters II	Parameters III
1·0	1·9433	1·8366	1·7821	1·9187	$a=20$	$a=90·6$	$\xi=-1·025$
0·316	1·8250	1·8358	1·7821	1·8383	$N=74·7$	$N=65·5$	$\lambda=0·9$
0·1	1·7000	1·7761	1·7816	1·6961			$N=104$
0·0316	1·4883	1·5260	1·6793	1·4791			
0·01	1·1525	1·1649	1·2030	1·1892			
0·00316	0·8942	0·8261	0·6637	0·8639			
$W\rightarrow$		4	4	3			
$\frac{n}{W}\Sigma(\bar{z}_i-Z_i)^2\rightarrow$		0·0705	0·3799	0·0123			
Variance ratio		1·487	8·015*	3·854			
D.F. n_1		4	4	63			
n_2		63	63	8			

* Significant ($P<0·1$ %); others not significant at $P=5$ %.

Table 6. *Comparisons of experimental with computed values for Exp. no.* 10

TMV conc. (g./l.)	$Y=\phi(x)\rightarrow$ $\bar{z}$	I (1*a*) Z	II (1*a*) Z	III (1*b*) Z	IV (2) Z	Parameters I	Parameters II	Parameters III	Parameters IV
1·0	2·4967	2·3691	—	—	2·4482	$a=47$	$a=59$	$a=253$	$\xi=-1·32$
0·316	2·3275	2·3691	2·2969	2·2398	2·3939	$N=234$	$N=198·5$	$N=172$	$\lambda=1·01$
0·1	2·2883	2·3583	2·2958	2·2398	2·2920				$N=320$
0·0316	2·2133	2·2603	2·2309	2·2386	2·1541				
0·01	1·9062	1·9895	1·9926	2·1153	1·9577				
0·00316	1·7592	1·6754	1·6877	1·6796	1·7268				
0·001	1·4958	1·4445	1·4533	1·3514	1·5083				
0·000316	1·3883	1·3265	1·3510	1·2688	1·3641				
$W\rightarrow$		6	5	5	5				
$\frac{n}{W}\Sigma(\bar{z}_i-Z_i)^2\rightarrow$		0·0936	0·0408	0·2296	0·0352				
Variance ratio		2·509*	1·094	6·155†	1·060				
D.F. n_1		6	5	5	80				
n_2		80	80	80	5				

* Significant at $P=5$ %.
† Significant at $P=0·1$ %. Others not significant.

The results of all the experiments to which the equation could be fitted, were those for which the inoculum was either crude sap or purified virus preparations at concentrations not exceeding 1 g./l. The equation was fitted on the assumption

that when the virus concentration was somewhere in the range 0·2–1·0 g./l., all the accessible susceptible regions developed lesions. Now, according to the assumptions from which equation (1 *a*) was derived, there is no gradation in susceptibility between susceptible regions, and only their number per half-leaf can vary. It follows that whenever a set of plants is inoculated with the same virus preparation, the maximum number of lesions should be obtained when the virus concentration reaches about 1 g./l. That this is not so, can be seen from the results of some of the experiments in which dilution-infection series were started from the virus concentration of 20 g./l.

Table 7. *Comparisons of experimental with computed values for Exp. no.* 13

TMV conc. (g./l.)	$Y=\phi(x)\rightarrow$ $\bar{z}$	I (2) Z	II (4) Z	Parameters I	Parameters II
20·0	2·3917	2·3560	2·3628	$\xi=0{\cdot}27$	$a_1=335$
4·0	2·3100	2·2780	2·3389	$\lambda=2{\cdot}16$	$N_1=59$
0·8	2·1217	2·1774	2·1403	$N=329$	$a_2=0{\cdot}63$
0·16	2·0275	2·0443	1·9648		$N_2=153$
0·032	1·9092	1·9121	1·9065		
0·0064	1·7908	1·7580	1·8527		
0·00128	1·5742	1·6100	1·5936		
0·000256	1·4817	1·4741	1·3696		
$W\rightarrow$		5	4		
$\frac{n}{W}\Sigma(\bar{z}_i-Z_i)^2\rightarrow$		0·0194	0·0682		
Variance ratio		1·474	2·385	Neither ratio is significant at $P=5\%$.	
D.F. n_1		80	4		
n_2		5	80		

Equation (2) could be fitted approximately to the results of all the experiments obtained in this work, so the set of assumptions from which it was derived may be true. Yet Lauffer & Price (1945) definitely reject these assumptions and accept those leading to equation (1 *a*). They base their conclusions on three lines of evidence. The first is that some of the published data on tobacco mosaic virus on *N. glutinosa* are sufficiently accurate to show that they fit equation (1 *a*) better than equation (2). They demonstrate this point graphically (Fig. 6 in their paper) with the results of two experiments selected from the literature, which they state fit equation (1 *a*) better than equation (2). The data did not fit equation (2) as well as they did equation (1 *a*) only because the values of the parameter N in equation (1 *a*) were adjusted to obtain a good fit to the data, and then the same values were used for the parameter N in equation (2). The parameter λ was taken as 0·5. A much better fit is obtained with different values for N and λ.

The second line of evidence given by Lauffer & Price is that 'the dilution curves of all viruses (of animals and plants) tend to have the same slope', notably the slope of the curve of equation (1 *a*). The slope of the curve of equation (1 *a*) is characteristic at each point corresponding to any particular

value of ax, whereas the slope of equation (2) is not characteristic at any particular point unless there is a reason for fixing the parameter λ at some particular value for all viruses, and there does not seem to be any such reason. However, it was because the slope of the line of experimental points could not be made to coincide with the curve of equation ($1a$) that equation ($1a$) could not be fitted to the data of many experiments with tobacco mosaic virus, whereas the curve of equation (2) could be fitted to the data of different experiments by substituting for λ different values considerably removed from 0·5, at which the slope of the curve most closely approaches that of the curve of equation ($1a$).

The third line of evidence used by Lauffer & Price comes from the study of local infections caused by mixed inocula. When a mixture of the related strains of tobacco mosaic and aucuba mosaic viruses is inoculated to *N. langsdorffi*, on which both form necrotic local lesions, some lesions appear to contain both strains, and some only one or the other. If the assumptions from which equation ($1a$) is derived are true, and if each virus strain can multiply freely in the presence of the other, the proportion of mixed infections can be predicted. If the two strains are mixed in equivalent proportions, i.e. if each strain, when present alone, forms approximately the same number of lesions, and if the parameters N and a in equation ($1a$) are adjusted so that the equation fits the results of dilution-infection series with the mixed inoculum, the proportion of lesions containing both strains obtained with a total virus concentration x will be expected to be given by the value of the infinite series

$$\rho=\frac{e^{-ax}}{1-e^{-ax}}\left\{\left[1-\frac{1}{2}\right]\frac{a^2x^2}{2!}+\left[1-\left(\frac{1}{2}\right)^2\right]\frac{a^3x^3}{3!}+\left[1-\left(\frac{1}{2}\right)^3\right]\frac{a^4x^4}{4!}+\ldots\right\}.$$

The experimental results obtained by Lauffer & Price differ widely from computed expectations, so that it seems strange that they should have considered them as evidence in favour of the assumptions on which the expectations are based. The computation is based on the assumption that each virus strain can multiply freely in the presence of the other, whereas there is much evidence to the contrary. If one virus strain is established in a host plant, it prevents the subsequent development of another related strain, and when two related strains are inoculated simultaneously, one can interfere with the formation of local lesions by the other (Sadasivan, 1940). Thus the presence of only one virus strain in a local lesion may be a result of suppression of development of the other strain. If so, the results obtained by Lauffer & Price cannot be considered as evidence for or against either of the two hypotheses under consideration.

The conclusion arrived at in this study, that the set of assumptions from which equation ($1a$) was derived must be false, does not exclude the possibility that the one virus particle in a susceptible region might produce a lesion. If equation ($1a$) is modified by changing the original assumptions or by introducing some auxiliary assumptions, it may then be fitted satisfactorily to the experimental results. One such modification, based on the assumption that virus particles are aggregated and dissociate on dilution, was introduced by

Bald (1937*c*). However, Bald's interpretation of the fact that the modified equation (1*a*) could be fitted satisfactorily to the experimental results, is that it 'is no proof that the kind of aggregation postulated in the derivation of the (modified) equation actually exists, and the constant K (present in the modified equation) must be considered as a measure of distortion of the dilution series rather than a measure of aggregation, unless an independent proof is obtained that the virus particles do aggregate in this manner'. Particles of tobacco mosaic virus can occur in linear aggregates, and the extent of such aggregation varies from one preparation to another depending mainly on the treatments to which the preparation has been subjected (Bawden & Pirie, 1945; Crook & Sheffield, 1946). There is no evidence that these aggregates dissociate on dilution, and no reason to assume that they would do so to the extent that would have to be assumed to make equation (1*a*) fit the experimental data. To fit equation (1*a*) to the results of Exp. no. 13, for example, it would have to be assumed that for the range of concentrations between 2·0 and 0·002 %, one virus aggregate is broken on average into 2·6 every time the solution was diluted 1:5. Also, as Fig. 5A shows, to fit equation (1*a*) to the results of different experiments, it is necessary to assume that the disaggregation caused by diluting the same virus preparation differs at different times.

The original assumptions from which equation (1*a*) was derived can be modified in other ways to fit the experimental results. Any modification that would make the parameter a vary suitably from one susceptible region to another, would serve this purpose. For example, it was originally assumed that the volume of inoculum that comes into contact with any susceptible region, is approximately constant, but if it is not, the value a will also vary. Alternatively, it can be assumed that there are u kinds of virus particles differing from one another in such a way that a particle of one kind can effectively infect any of u kinds of susceptible regions, a particle of the second kind can infect any of $(u-1)$ kinds of regions, a particle of the third kind any of $(u-2)$ kinds of regions, and so on. Any of these assumptions would modify equation (1*a*) into the form

$$Y = \sum_{i-1}^{u} N_i (1 - e^{-a_i x}).$$

In fact, if only two different values for N and a are postulated, so that equation (1*a*) will be modified into equation (4), it could be fitted satisfactorily to the results of all the experiments obtained in this work by suitably adjusting the values of the four parameters N_1, a_1, N_2 and a_2. This was shown statistically for Exp. 13. The fact that adding various auxiliary assumptions to the hypothesis, that infection can be caused by single virus particles, can make it compatible with experimental results, proves nothing, but it does make it possible that the hypothesis may be true.

Equation (2) could be approximately fitted to the results of all the experiments obtained in this work. Thus the hypothesis of variation in susceptibility of susceptible regions, with logarithms of minimal effective concentrations normally distributed, may be true. The fact that no auxiliary assumptions are needed may be considered as an argument in favour of the hypothesis. Any

conclusive evidence is to be expected from some new experimental approach to the problem rather than from any mathematical treatment of the information at present available.

In conclusion it should be emphasized that there is no reason to suppose that all infective agents referred to as 'viruses' would display the same basic mechanism of infecting their hosts, as it is often assumed in discussions on the subject.

REFERENCES

Bald, J. G. (1937*a*). The use of numbers of lesions for comparing the concentrations of plant virus suspensions. I. Dilution experiments with purified suspensions. *Ann. appl. Biol.* **24**, 33.

Bald, J. G. (1937*b*). The use of numbers of lesions for comparing the concentrations of plant virus suspensions. II. Distortion of the dilution series. *Ann. appl. Biol.* **24**, 56.

Bald, J. G. (1937*c*). The use of numbers of lesions for comparing the concentrations of plant virus suspensions. IV. Modification of the simple dilution equation. *Austr. J. exp. Biol. med. Sci.* **15**, 211.

Bawden, F. C. & Pirie, N. W. (1945). The isolation and properties of tobacco mosaic virus in different states of aggregation. *Brit. J. exp. Path.* **26**, 294.

Bliss, C. I. (1935). The calculation of the dosage-mortality curve. *Ann. appl. Biol.* **22**, 134.

Bryan, W. R. & Beard, J. W. (1940). Host influence on the characterization of response to the papilloma protein and to vaccinia virus. *J. infect. Dis.* **67**, 5.

Crook, E. M. & Sheffield, F. M. L. (1946). State of aggregation of tobacco mosaic virus. *Brit. J. exp. Path.* **27**, 328.

Finney, D. J. (1947). *Probit Analysis.* Cambridge University Press.

Gaddum, J. H. (1933). Methods of biological assay depending on a quantal response. *Spec. Rep. Ser. Med. Res. Coun.* no. 183. London: H.M. Stationery Office.

Haldane, J. B. S. (1939). Sampling errors in the determination of virus density by the dilution method. *J. Hyg., Camb.*, **39**, 289.

Kleczkowski, A. (1949). The transformation of local lesion counts for statistical analysis. *Ann. appl. Biol.* **36**, 139.

Lauffer, M. A. & Price, W. C. (1945). Infection by viruses. *Arch. Biochem.* **8**, 449.

Parker, R. F. (1938). Statistical studies of the nature of the infectious unit of vaccinia virus. *J. exp. Med.* **67**, 725.

Sadasivan, T. S. (1940). A quantitative study of the interaction of viruses in plants. *Ann. appl. Biol.* **27**, 359.

Youden, W. J., Beale, H. P. & Guthrie, J. D. (1935). Relation of virus concentration to the numbers of lesions produced. *Contr. Boyce Thompson Inst.* **7**, 37.

(*Received* 30 *April* 1949)

The Assay of the Antibiotic Nisin

By A. HIRSCH
National Institute for Research in Dairying, University of Reading

SUMMARY: Nisin, which does not diffuse quickly through agar, may be assayed by dilution methods; or, because it is bactericidal and not merely bacteriostatic, it may be assayed by estimating numbers of surviving bacteria.

Nisin in low sublethal concentration simply prolongs the lag-phase of *Streptococcus agalactiae*; in higher sublethal concentrations it also induces fast-growing resistant strains. In both cases the delay can be accurately related to nisin concentration, growth being measured in terms of acid production.

The available methods for the assay of nisin are limited by the properties of the substance.

Nisin does not diffuse quickly through agar at pH 7 (Mattick & Hirsch, 1947), a character shared (Heatley, 1947) with the allied antibiotic diplococcin (Oxford, 1944). Assay by the agar-diffusion technique (Heatley, 1944) can be very accurate, and various modifications of it have been used for penicillin and streptomycin (Foster & Woodruff, 1943; Foster & Wilker, 1943; Brownlee, Delves, Dorman, Green, Grenfell, Johnson & Smith, 1948). Subtilin appears to be a borderline case since Lewis, Humphreys, Thompson, Dimick, Benedict, Langlykke & Lightbody (1947) recommend its assay by turbidimetric means, whereas Housewright, Henry & Berkman (1948) use agar-diffusion methods. According to MacMorine & Slinn (1948) subtilin does diffuse slowly through agar and if the growth of the test-organism is retarded plate assays become possible. This technique has not yet been explored with nisin, which in turbidimetric assay has an unfavourable range.

Various methods of assay for nisin were investigated, within the limitations described. Nisin solutions containing 7·5 mg./ml. (about 10,000 units/ml.) can be assayed as surface-active cationic material. Epton's (1947) method was tried but was discarded when it was found that small concentrations of nisin could not be assayed and that impurities caused marked errors.

A dilution end-point method of assay was supplemented by a methylene-blue test. As early as 1934, Cox recommended the use of methylene blue for the qualitative detection of inhibitory organisms in milk, and similar methods have been used for the assay of penicillin and streptomycin (Reid & Brewer, 1946; Sanchez & Lamensans, 1947). With nisin this test allows a reading in about 20 min. and has obvious advantages. These tests were superseded by a test based on the bacterial power of nisin and finally by the 'lag-phase assay'.

The accuracy of the dilution method and the methylene-blue test depends on the factor used in making serial dilutions. Geometrical series are usually set up, although arithmetical series (Schmidt & Moyer, 1944) are sometimes used. In the usual statistical terms the error ($\pm t \times$ standard error of the potency ratio) of a dilution method cannot be defined (Healy, 1948). The

uncertainty, however, when dealing in powers of two is ±50 %, not allowing for experimental error. The error of assay by agar-diffusion techniques is usually ±10 to ±15 %, and it has been the aim of this work to achieve comparable accuracy. The bactericidal test has a mean accuracy of ±32 % and the lag-phase assay one of ±10 %.

METHODS

Cultures. For the rapid methylene-blue test a fast-growing strain of *Streptococcus cremoris* (1 P 5) was subcultured daily in sterile milk using a 1 % (v/v) inoculum and incubation at 22°. Before a test was begun a 10 % (v/v) inoculum of the daily subculture was made in warm milk and incubated at 30° for 1 hr. (referred to subsequently as 1 P 5 milk). This culture will reduce methylene blue in about 1 min. and will clot after 1 hr. at 30°.

For all other assays the test-organism was a haemolytic strain of *Str. agalactiae.* It was subcultured daily and the fresh subculture stored in the refrigerator during working hours and incubated at 37° overnight. The organism was replaced once a month from a stock culture. Occasionally fresh cultures were started from freeze-dried material.

Media. Separated milk with or without added chalk and sterilized by steaming on three successive days was used for the propagation and maintenance of *Str. cremoris* (1 P 5).

Glucose Lemco broth (Mattick & Hirsch, 1947) was used for the propagation of *Str. agalactiae* and bullock heart medium for storage.

The assay media were as follows:

(*a*) Glucose Lemco broth for the dilution-method assay.

(*b*) For the bactericidal test 0·5 % horse blood agar at pH 8·0.

(*c*) For the lag-phase assay a medium containing $Na_2HPO_4.2H_2O$, 1 part; NaCl, 1 part; glucose, 2 parts; peptone (Evans'), 2 parts; Na acetate (hydrated), 4 parts was used. These were dried, pulverized and blended, and the dry powder stored in the refrigerator. When required, 50 g. of powder were mixed with 1 l. of distilled water and sterilized at 10 lb. for 10 min. The pH was adjusted with N-NaOH. Evans's peptone gave heavier growth than British Drug Houses Ltd., Benger's, Bacto- and Neo-peptones.

Methods of counting. Plate counts and the roll-tube method were used (Hirsch, 1948).

Methylene-blue solution was prepared by dissolving one tablet (standard tablet, British Drug Houses Ltd.) in 200 ml. of water and stored in a dark bottle; 0·3 ml. of this solution was used per 10 ml. milk.

Units of nisin have already been defined by Mattick & Hirsch (1947).

Standards. The standard preparation of nisin was a batch of powder stored at −25°. Fresh standard solutions containing 10,000–50,000 units/ml. were prepared monthly. A solution in 0·02 N-HCl was made and sterilized by placing in a boiling water-bath for 5 min. This solution when stored at 0–2° deteriorated only slowly. Dilute solutions required for assay are, however, unstable and cannot be stored.

Samples for assay were acidified with N-HCl and if necessary sterilized by placing in a boiling water-bath for 5 min. At pH 3 nisin is heat stable and small quantities of acid have a negligible effect on the assay.

Diluent. The diluent for the dilution and bactericidal assays was acidified distilled water or saline. For the lag-phase assay the standard diluent used was glucose Lemco-broth in which *Str. cremoris* (1 P 5) had grown overnight, the cells being then removed by centrifuging and the supernatant broth sterilized by steaming.

Cleaning of glassware. Nisin is adsorbed on glass and traces of it are extremely difficult to remove. Test-tubes thus contaminated may yield up to 2 units of nisin per ml. when sterile fresh broth is placed in them. Two consecutive boilings in the detergent 'Hexo' (Boro' Dairy Laboratories, London) will usually destroy residual nisin, but even after this procedure it is advisable to test each batch of test-tubes for growth inhibition.

For the preparation of master solutions in the lag-phase assay it is advisable to use new test-tubes cleaned in chromic-sulphuric acid mixture.

DILUTION METHODS OF ASSAY

The procedure with *Str. agalactiae* has been described by Mattick & Hirsch (1947).

For the rapid methylene-blue assay with *Str. cremoris* (1 P 5), the following procedure was adopted:

Unknown solution (ml.)	1·00	0·50	0·30	0·20	0·13	0·10
Distilled water (ml.)	0	0·50	0·70	0·80	0·87	0·90
1 P 5 milk (ml.)	9	9	9	9	9	9
Final dilution	1/10	1/20	1/33	1/50	1/77	1/100

After the addition of the 1 P 5 milk the tubes were placed in a 30° water-bath for 10–15 min. The methylene blue was then added and the tubes were inverted to mix and reincubated for a further 5 min. when the colour of tubes containing sufficient nisin for the inhibition of the test organism was unchanged, whereas the other tubes were colourless. *Str. agalactiae* and *Mycobacterium phlei* both behave erractically towards methylene blue.

Although *Str. agalactiae* is about four times less sensitive to nisin than *Str. cremoris* (1 P 5), there was reasonable concordance between the two dilution-method assays as shown in Table 1.

Table 1. *The reproducibility of methylene-blue assay and its concordance with the dilution end-point assay*

Sample	Units/ml. Methylene-blue assay	Units/ml. Dilution end-point assay
1	60, 60, 40	—
2	100, 120, 100	—
3	150, 150, 133	—
4	100, 100	100
5	233, 200	200
6	150, 133	100
7	60, 100	80
8	120	100

THE BACTERICIDAL METHOD OF ASSAY

The survival of Streptococcus agalactiae *in solutions of nisin.* The bacterial death-rates with nisin are typical of a disinfectant (Hirsch & Mattick, 1949). Since it is usually possible to arrive at a simple linear relationship between the concentration of a disinfectant and its biological activity, it was hoped that this would facilitate the assay of nisin. However, since *Str. agalactiae* usually grows in chains, there was doubt about the possibility of an accurate count. Contrary to expectation, it was found that the strain of *Str. agalactiae* used could be counted with considerable accuracy. This can probably be attributed to the fact that the old laboratory culture used occurs largely in the form of diplococci, and that the experiments were carried out by one observer only. The control chart of Hannay (1946) was used. Eighty-eight sets of replicate colony counts showed that with four degrees of freedom $x^2 = 5{\cdot}917$ and the probability of an agreement between expected and observed readings is within 10 %.

The data were insufficient for a critical assessment of the true nature of the dose-response curve. Approximate linear relationship was given by a number of functions. Thus the logarithm of concentration could be used either against the logarithm of percentage survivors or against probits, but the logarithm of percentage survivors could also be plotted against nisin concentration. These three cases are illustrated with the data of one experiment in Fig. 1. Besides these, however, other possibilities also existed, but despite these apparently contradictory results the impression was formed that the true linear relation was between the logarithm of concentration and probits, as suggested by Withell's work (1942*a*, *b*).

The procedure for the bactericidal assay

In preliminary experiments it was established that the range which could be covered by the assay was always about fivefold. When the cell concentration was about 300,000/ml. an average of 2–10 units of nisin/ml. could be assayed. This cell concentration was chosen for further work, since the cells were not too numerous to make dilutions for plating unduly laborious. One difficulty was the elimination of carried-over nisin, since 0·4 unit/ml. has a marked bactericidal action when the number of cells is below 5000/ml. The count usually entailed plating dilutions from 1/100 to 1/1000, thus rendering the effect of carry-over negligible. As nisin is alkali-labile, the high pH of the plate medium was an added precaution.

The procedure was as follows. A 24 hr. culture of *Str. agalactiae*, containing 200–400 million viable particles per ml. was diluted 100-fold and 1 ml. added to 8 ml. of sterile glucose Lemco broth (contact tube); 1 ml. quantities of the 20–100 units/ml. nisin solutions were added to the contact tubes. At least two standard solutions and one control containing no nisin were set up with each assay. The two nisin standards were usually 2 and 10 units/ml. to give about 80 and 10 % survivors respectively.

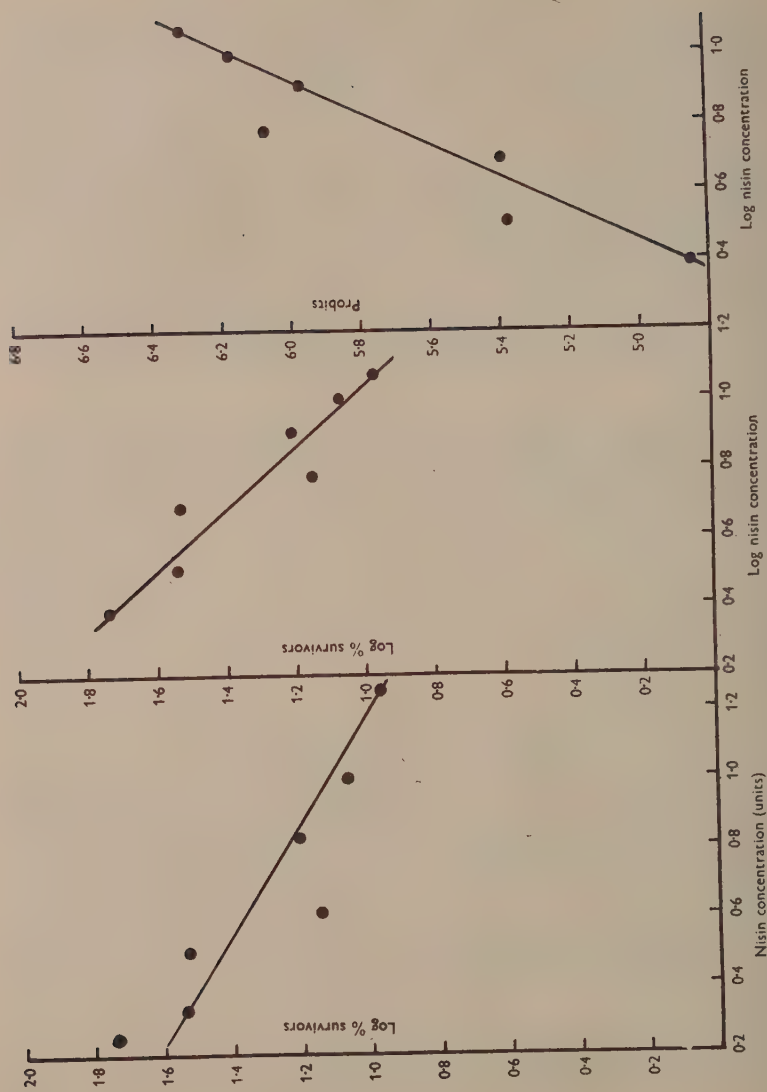


Fig. 1. Some dose-response curves for the bacterial assay of nisin.

The inoculated contact tubes were placed in a water-bath accurately controlled at 37°. After 5 min. necessary to bring the tubes to 37°, 1 ml. of the nisin solutions and the control saline were added every 1½ min. from 0 to 9 min. (seven solutions, three controls and four unknowns). After 10 min. the first contact tube was sampled; from 10 ml. of medium samples were withdrawn with 1 ml. pipettes. When the count fell to below 20,000 colonies/ml. there was no correlation between loop and pipette counts. During the next 1½ min. a series of 10-fold dilutions up to 1/10,000 was made. The blood-agar was poured on the plates while the next series was warming to 37°. The plates were counted after 24 hr. incubation at 37°.

Using the four-point assay design of Bliss (1944) on the data of one experiment the accuracy of this method was determined. A nisin solution containing 7·2 units/ml. was assayed as an unknown against another standard, and with only one sample or only one plate per sample results from 5·1 to 8·6 units/ml. for the solution were obtained. Under the most favourable conditions (three samples each with three plates) the estimated potency of this solution was 7·04 units/ml. with approximate 95 % limits of ±2·2 units or ±32 % ($P=0{\cdot}95$).

When this assay is made as accurately as possible, it is more accurate than the dilution end-point method, but when only one sample of the unknown is tested, even with replication, its accuracy is approximately that of the dilution end-point method.

THE LAG-PHASE METHOD OF ASSAY

The growth of Streptococcus agalactiae *in the presence of small concentrations of nisin*

The observation that with prolonged incubation the titres obtained in a dilution end-point method of assay decrease, suggested a relationship between the time required for a culture to start growing and nisin concentration. At the end-points of dilution assays the concentration of nisin is constant, irrespective of the original strength of the sample and thus this relationship suggested itself for assaying. It was realized that a measurement of time was difficult to make as a routine. Instead, pH measurements were taken, since the amount of acid produced by the organisms in a buffered medium is related to the time when growth began. An approximately linear relationship was found between pH and the logarithm of the nisin concentration.

The assay technique first used thus consisted in plotting a standard line and reading off the nisin concentration from this (Fig. 2). Wood (1946) gave the name of 'single curve' method to this assay technique which does not involve the plotting of a curve for the unknown. The accuracy of this method was tested by assaying one standard solution, divided into two parts, and expressing the activities of the solutions in terms of each other. Discrepancies up to 30 % were obtained and it was felt that, apart from the unfavourable slope of the standard line, some other factors must be affecting the assay.

Further observations showed that when the curves were divided into two portions a statistically significant increase in accuracy was obtained.

Comparisons between the ranges 1–5 and 5–50 units were not possible without loss of accuracy. The standard curve as shown in Fig. 2, where each point is the average of sixty-three readings, was found to be heterogeneous, i.e. the responses in the two ranges (1–5 and 5–50 units/ml.) were due to different causes.

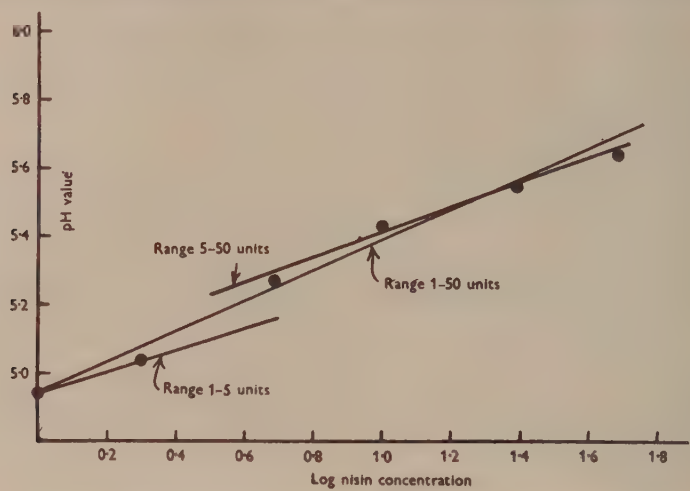


Fig. 2. The heterogeneous response of *Str. agalactiae* in the lag-phase assay.

Table 2. *The effect on the production of resistant cultures by growth of* Streptococcus agalactiae *in various concentrations of nisin*

Organism grown in nisin (units/ml.)	Strain no.	*Organisms at the end of growth, inhibited by: Units/ml.	Average units/ml.
0	1	1	1
	2	1	
	3	1	
1	1	1	1
	2	1	
	3	1	
2	1	1	1
	2	1	
	3	1	
5	1	20	9
	2	2	
	3	5	
10	1	10	23
	2	50	
	3	10	
20	1	50	27
	2	10	
	3	20	
100	1	50	40
	2	20	
	3	50	

* Sensitivity test carried out by a dilution end-point method.

Experiments were therefore carried out in which the pH of inoculated broths containing varying amounts of nisin was measured at intervals of time. From Fig. 3 it can be seen that growth of the normal culture (0) is delayed, and the length of lag is related to the nisin concentration. The resulting straight lines

Fig. 3. The effect of nisin on the acid production of *Str. agalactiae*.

are, however, of different slopes; in all cases the eventual growth of those cultures which contain nisin is much more rapid (curves 1, 2). All the cultures appeared to be stimulated by nisin, but from 5 to 50 units/ml. the curves obtained converged. This observation has been repeatedly made. An explanation was required and accordingly the sensitivity to nisin of the test organisms at the end of the assay was tested. From Table 2 it can be seen that 1 and 2 units/ml. did not produce resistant organisms; other experiments have shown that this is true of up to 4–4·5 units/ml. Concentrations greater than 5 units/ml. invariably yielded nisin-resistant organisms at the end of the assay.

The range 1–5 units of nisin/ml. may thus be regarded as causing a true increase in the length of the lag-phase. In the range of 5–10 units/ml. secondary

growth appears, which, because nisin is bactericidal, is undoubtedly preceded by the killing of a large proportion of sensitive cells. This point was confirmed by plate counts. It appears that, proportionally with increasing nisin concentration, increasingly resistant cultures result which, once growth commences, grow faster than the sensitive cells.

When samples taken at 30 min. intervals were examined it was found that the sensitivity of the few cultures which could be isolated at the beginning did not change during the experiment; from the start only resistant forms could be isolated from tubes containing 75 units/ml. It thus appears that there is a selection of naturally occurring resistant cells.

The observations recorded in Fig. 3, in which the amount of stimulation appeared to be proportional to the nisin concentration, suggested that the resistant cells might utilize nisin. Accordingly, resistant and normal cultures were grown in the presence of various concentrations of nisin, the optical densities and the pH were measured hourly. Contrary to expectation it was found that nisin did not now stimulate growth, but whereas the growth of normal cultures in nisin was delayed, resistant cultures grew in the presence of nisin at the same rate as the controls without nisin.

The line T_3–T'_3 in Fig. 3 represents the moment when the experiment is stopped and pH readings taken. It is obvious that this moment has to be carefully selected in order to obtain maximum accuracy.

Choice of dose-response relationship

The response due to the formation of resistant cultures (approximate assay range 5–50 units/ml.) was preferred to the lag-phase response (range of 1–5 units/ml.), not only because of the range, but also because of the more favourable slope. The following convenient linear relationships exist in this case:

(*a*) Range of 5–15 units: units × pH = a constant.

(*b*) Range of 5–50 units: log of units × pH = a constant.

(*c*) Range of 5–50 units: log lag period × pH = a constant.

Titrations of the acid formed, instead of pH measurements, were tried and discarded. They were no better than pH readings and were more laborious.

The lag period is determined quite simply when two pH readings are taken at intervals of time. It was found that the logarithm of the time is linearly related to pH, so that the line joining the two pH readings may be extrapolated and the length of the lag determined. Cases (*a*), (*b*) and (*c*) are shown in Fig. 4.

Whereas case (*a*) appears to be the most accurate, the range is very small. Occasionally data were obtained, which for no apparent reason, could not be fitted to case (*c*). The preferred relationship was therefore that of case (*b*), but before further experiments could be usefully undertaken it was necessary to test the effect of impurities on the assay. Nisin of varying purities, prepared from culture fluids with and without various peptones were used. The results presented in Fig. 5 show that the response is specifically due to nisin since the same slopes were obtained. Further experiments were now undertaken on the preferred method.

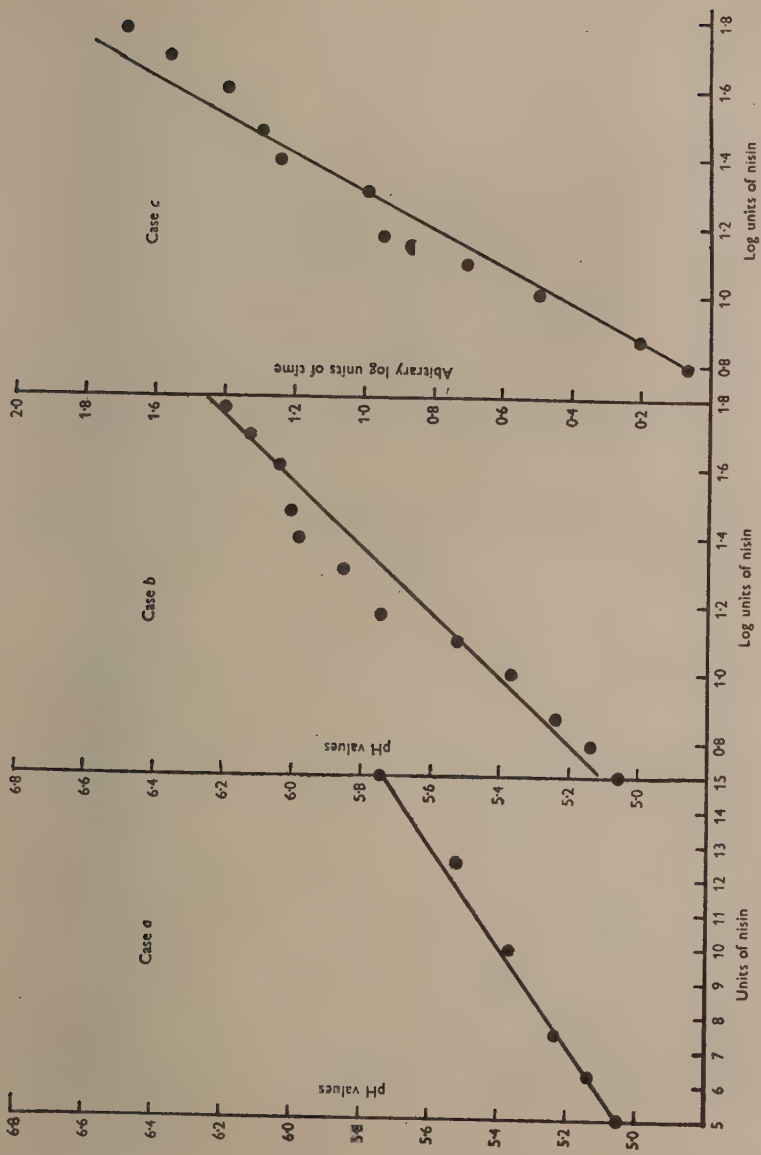


Fig. 4. Some dose-response curves for the lag-phase assay of nisin.

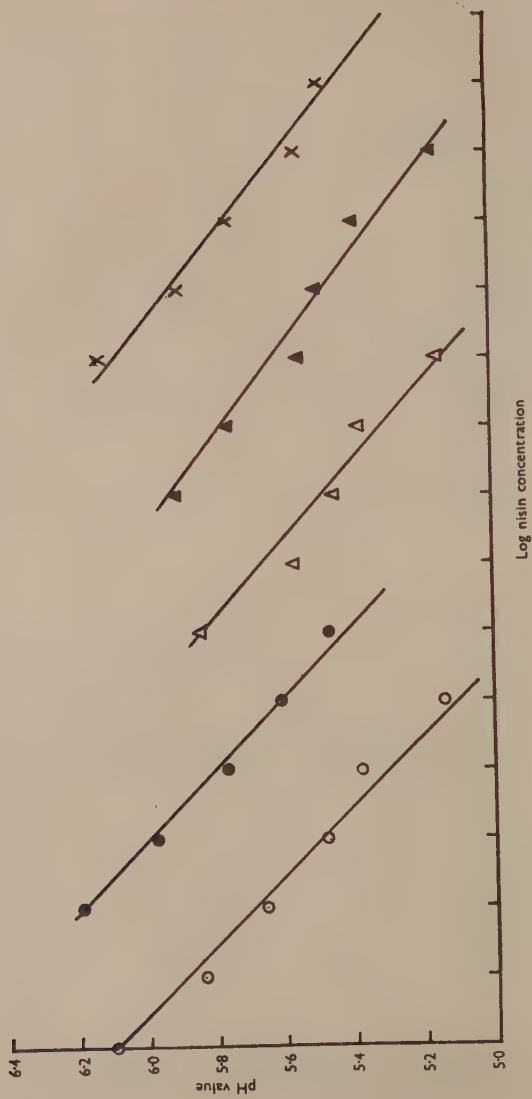


Fig. 5. The effect of impurities on the assay of nisin by the lag-phase method. × = crude nisin made from peptone medium; ▲ = crystalline nisin; △ = nisin preparation made from peptone medium in 1946; ● = nisin preparation made from peptone medium in 1948; ○ = nisin preparation made from yeast medium.

Variables of the preferred method

Apart from improvement in accuracy, one of the reasons which prompted investigation of the preferred method was that when two identical standard solutions were assayed, the responses did not always coincide. The causes of this disconcerting effect may be summarized as follows:

(*a*) The most important single factor is the age of the test-organism. When the inoculum was 6–10 hr. old instead of 18–24 hr. much better slopes were obtained (cf. Figs. 2, 5), the upper range of linearity was extended from 50 to 100 units/ml. and the accuracy and usefulness of the technique much improved.

(*b*) The nature of the diluent used was important. Markedly different slopes, suggesting almost that different substances were being assayed, were obtained when the standard was made up in distilled water instead of broth.

(*c*) A progressive shift of the standard line was observed with assays lasting more than a few hours, those standards which were kept longer usually giving a lower reading.

(*d*) The method of preparing the dilution was of slight importance; the number of pipettes used affected the answer but slightly. The temperature of the diluent was unimportant.

(*e*) Fatty acids from cotton-wool plugs did not appear to affect the assays. Cleanliness of the glassware was of prime importance and the critical master dilution tubes (see below) had to be scrupulously cleaned. The test-tubes had also to be of even size.

Method of assay

(*a*) *Preparation of the standard.* A solution containing 10,000–30,000 units of the standard/ml. was serially diluted in 10 ml. lots of diluent, preferably using a fresh pipette for each concentration. The concentrations were ten times higher in these master dilutions than in the final tubes. Each dilution was then distributed in 1 ml. lots using only one pipette to make ten replicates for each concentration. In routine practice the method is sufficiently reproducible to make it unnecessary to use more than four tubes for each concentration. To the tubes containing 1 ml. of nisin 9 ml. of inoculated broth were added as rapidly and accurately as possible.

(*b*) *Preparations of unknowns.* Solutions of chemically concentrated nisin preparation are not difficult to assay. They may be sterilized by heat and then diluted. Culture fluids containing nisin are, however, very much more difficult to assay. According to various treatments the same culture fluid can give different answers. To obtain consistent results it is necessary first to adjust to pH 3·0. The broth is then boiled to extract more nisin from the cells, which are then centrifuged down. Filtration through Seitz-type filter-pads is not suitable as nisin is adsorbed by the filter-pads.

The choice of assay design

Wood (1946) and Wood & Finney (1946) have defined the best assay design for microbiological assays of growth substances and there seems no reason why their definitions should not hold for the assay of antibiotics; the principles

involved are the same. The upper end-point of the graded response is that concentration of the antibiotic at which growth is no longer possible. In the same way in microbiological assays, the lower end-point is that concentration of the essential nutrients which does not permit growth. The most accurate design is the 'common-zero 5-point' assay. They recommend 'that, if the linear relationship does not ordinarily hold down to zero dose for the particular assay procedure in use, a small quantity of the factor being assayed is added to the basal medium for all tests, in order to make the relationship linear...'. Since in the preferred method of assay the linear relationship ends at concentrations less than 5 units/ml., experiments were carried out in which the tubes contained 5 units added nisin per ml. The results were not encouraging, probably because contact between nisin and cells in the inoculated broth lead to an unpredictable condition of the inoculum.

The remaining practicable design is that of parallel lines. Whereas for the same amount of labour this method is less precise than the 'common-zero 5-point' assay the experimental arrangement is easier and the parallelism of the lines indicates that the response to standard and unknown are due to the same substance. It is thus possible to obtain fairly reliable assay results by simple graphical procedures as suggested by Wood. It is important that the dilutions should be prepared as geometrical series so that when a plot of log nisin concentration against pH is made, an equally weighted line is obtained.

Bliss (1944) described a simplified method of calculation for the assay of penicillin. Later he developed graphic control charts which he used for the assay of penicillin (Bliss, 1946). By comparison with nisin, the penicillin assay is a well-established method, and it was felt that the 4-point assay design was not suitable for this comparatively new substance. A 6-point assay which could test curvature as well as parallelism was preferable, and Healy (1949) has developed this method to enable its use in routine assay. Applying this method to data collected over a period of time the accuracy of the lag-phase assay varies from ± 7 to $\pm 15\%$.

I wish to thank Dr A. T. R. Mattick for his encouragement and helpful criticism throughout the course of this work: Dr N. J. Berridge and Mr M. J. R. Healy, for useful advice; and Messrs Benger's Ltd. for gifts of nisin.

REFERENCES

Bliss, C. I. (1944). A simplified calculation of the potency of penicillin and other drugs assayed biologically with a graded response. *J. Amer. Statist. Ass.* **39**, 479.

Bliss, C. I. (1946). A revised cylinder-plate assay for penicillin. *J. Amer. pharm. Ass.* **35**, 6.

Brownlee, K. A., Delves, C. S., Dorman, M., Green, C. A., Grenfell, E., Johnson, J. D. A. & Smith, N. (1948). The biological assay of streptomycin by a modified cylinder-plate method. *J. gen. Microbiol.* **2**, 40.

Cox, G. A. (1934). A simple method for the detecting of 'non-acid' milk. *N.Z. J. Agric.* **49**, 231.

Epton, S. R. (1947). A rapid method of analysis for certain surface-active agents. *Nature, Lond.*, **160**, 795.

FOSTER, J. W. & WILKER, B. L. (1943). Microbiological aspects of penicillin. II. *J. Bact.* **46**, 377.

FOSTER, J. W. & WOODRUFF, H. B. (1943). Microbiological aspects of penicillin. I. *J. Bact.* **46**, 187.

HANNAY, C. L. (1946). A control chart for bacterial colony counts. *Proc. Soc. appl. Bact.* no. 2, p. 85.

HEALY, M. J. R. (1948). Private communication.

HEALY, M. J. R. (1949). Routine computation of six-point biological assay. *Biometrics* (in the Press).

HEATLEY, N. G. (1944). A method for the assay of penicillin. *Biochem. J.* **38**, 61.

HEATLEY, N. G. (1947). A simple plate method for multiple tests on the antibacterial activity of many bacteria against other bacterial strains. *J. gen. Microbiol.* **1**, 168.

HIRSCH, A. (1948). An apparatus made from Meccano parts for the preparation of Roll-tube cultures. *J. gen. Microbiol.* **2**, 123.

HIRSCH, A. & MATTICK, A. T. R. (1949). Some recent applications of nisin. *Lancet*, ii, 190.

HOUSEWRIGHT, R. D., HENRY, R. J. & BERKMAN, S. (1948). A microbiological method for the assay of subtilin. *J. Bact.* **55**, 545.

LEWIS, J. C., HUMPHREYS, E. M., THOMPSON, P. A., DIMICK, K. P., BENEDICT, R. G., LANGLYKKE, A. F. & LIGHTBODY, H. D. (1947). The microbiological assay of subtilin. *Arch. Biochem.* **14**, 437.

MACMORINE, H. G. & SLINN, G. S. (1948). Plate techniques for the assay of subtilin. *Canad. J. Publ. Hlth*, **39**, 203.

MATTICK, A. T. R. & HIRSCH, A. (1947). Further observations on an inhibitory substance (nisin) from lactic streptococci. *Lancet*, ii, 5.

OXFORD, A. E. (1944). Diplococcin, an antibacterial protein elaborated by certain milk streptococci. *Biochem. J.* **38**, 178.

REID, R. D. & BREWER, J. H. (1946). The reductase method for the determination of penicillin concentrations in body fluids. *J. Bact.* **52**, 251.

SANCHEZ, G. & LAMENSANS, A. (1947). Méthode de dosage rapide de la pénicilline et de la streptomycine. *C.R. Acad. Sci., Paris*, **224**, 1189.

SCHMIDT, W. H. & MOYER, A. J. (1944). Penicillin. I. Methods of assay. *J. Bact.* **47**, 199.

WITHELL, E. R. (1942*a*). The significance of the variation in shape of time-survivor curves. *J. Hyg., Camb.*, **42**, 124.

WITHELL, E. R. (1942*b*). The evaluation of bactericides. *J. Hyg., Camb.*, **42**, 339.

WOOD, E. C. (1946). The theory of certain analytical procedures with particular reference to microbiological assays. *Analyst*, **71**, 1.

WOOD, E. C. (1947). The computation of microbiological assays of amino-acids and other growth factors. *Analyst*, **72**, 84.

WOOD, E. C. & FINNEY, D. J. (1946). Design and statistical analysis of microbiological assays. *Quart. J. Pharm.* **19**, 112.

(*Received* 21 *May* 1949)

Some Aspects of Cell Division in *Saccharomyces cerevisiae*

By A. A. BARTON
The Distillers Company Ltd., Research and Development Department, Great Burgh, Epsom, Surrey

SUMMARY: Two types of scar are formed on yeast cells as a result of the division process: a single 'birth scar' marking the point at which the cell was joined to the parent, and a variable number of 'bud scars' marking the points at which buds have been formed. A regular sequence of bud formations occurs. Cultures grown from the first and last buds appear to be identical.

Interest in the process of budding in yeast cells has centred round the behaviour of cell inclusions. Nevertheless, reproduction by the formation of buds is a cytokinetic process of some interest to cytologists. With a view to extending knowledge of this subject the cell division of a strain of *Saccharomyces cerevisiae* was investigated.

METHODS

The strain of yeast used was D.C.L. baking yeast, a single-cell strain identified as *Saccharomyces cerevisiae* Hansen var. *ellipsoideus*. This was maintained at 30° in a 15% (w/v) aqueous solution of malt extract (final sp.gr. 1050) and transfers made every 48 hr. Observations were made on living and non-living material. Stained material was mounted in Canada balsam after careful dehydration through a series ethanol-water mixtures of increasing ethanol concentration.

The optical system consisted of Zeiss 2 and 4 mm. apochromatic objectives together with ×10 and ×20 Zeiss Compens eyepieces. Illumination was provided by a 30 c.p. Ediswan Pointolite lamp or a high-pressure mercury-vapour lamp in conjunction with a Watson-Conrady condensing lens and a Wratten 38A or an Ilford 601 filter.

Scars on yeast cells

A study of the process of cell division reveals that the disconnexion of a cell from its bud produces characteristic scars on the wall of both cells. In a nutrient medium the scars on a yeast cell (see 'bud scars' below), are angular projections from the cell wall (Pl. 1, fig. 1), but the similarity in refractive index of cell wall and surrounding fluid precludes further study. The necessary difference in refractive index can be obtained by the transfer of single cells on to a somewhat dry film of agar by a micromanipulator. The cell wall then lies in contact with moist air on the side away from the agar and this, possibly combined with a slight drying of the cell surface, reveals the scars in greater detail (Pl. 1, fig. 2). Each scar is seen to consist of a slightly raised circular rim, approximately 2 μ in diameter, enclosing a centrally thickened plug. This marks the last point of cytoplasmic connexion between the mother and daughter

cells. The size and shape of each scar corresponds to that of the neck, or channel, which originally joined the two cells.

In mature yeast cells stained by the Newton crystal violet method, after fixation in osmium tetroxide vapour (Baker, 1945), two types of scar can be demonstrated (Pl. 1, fig. 3): a single scar, termed the 'birth scar', marking the point at which the cell, as a bud, was connected with the parent cell; and a varying number of bud scars, which mark the positions at which buds have been formed. One of these is shown in Pl. 1, fig. 3. Another method for the study of scars is plasmolysis of the cells by heating to *c.* 60°. This is a particularly useful technique when microscopic resolution is increased by photographing the cells under monochromatic mercury-violet illumination (4359 A.) (Pl. 1, fig. 4).

The 'birth scar' is invariably situated at a point central to the long axis of the cell (Pl. 1, figs. 3 and 4), and can be distinguished from bud scars, which it resembles in the unplasmolysed condition, by a resistance to collapse when the cell is fixed and stained (Pl. 1, fig. 3).

Before separation the rims of the two scars of both cells are believed to lie in contact, strengthening the bond between the cells. When, however, the cytoplasmic connexion is lost, the independent increase in volume of the daughter cell results in a stretching of its birth scar; the shearing action between the two scars then causes the mechanical connexion to break. One consequence is that the birth scar has a greater diameter (3 μ) than the other scars. Any bud scar produced before the maximum size of the cell has been attained will also be stretched, though not to the same extent as the birth scar (Pl. 1, fig. 4).

Site of bud formation

By means of a micromanipulator, the successive buds formed from single cells growing on malt-extract agar were removed as soon as they became free of the parent cell. By using the position of undetached buds as topographical markers the sites of origin of buds one to eight was noted. The points of origin, reduced to two dimensions, are shown in Pl. 1, fig. 5. This positioning was found to be constant in the fifteen cells observed. A bud was never seen to form at the site of a scar.

Effects of age on the cell

A yeast cell was observed to bud twenty-three times. Microscopically, cultures grown from the first and last buds were identical. In the two cases examined, the yield of pressed yeast from a fixed amount of molasses and the dough-raising capacity of the yeast produced were the same for cultures grown from the first and the fifteenth buds of the same cell.

Any tendency to degeneration comparable with that described by Schouten (1935) in yeast was not observed.

The author thanks the Directors of the Distillers Company Ltd. for permission to publish this paper.

REFERENCES

Baker, J. R. (1945). *Cytological Technique*, 2nd ed. London: Methuen and Co. Ltd.

Schouten, S. L. (1935). Altersdegeneration an Hefezellen. *Arch. exp. Zellforsch.* **17**, 451.

EXPLANATION OF PLATE

Fig. 1. Scars (marked *X*) on the cell wall of a yeast cell growing in malt-wort. Wratten 38 A filter; × 3600.

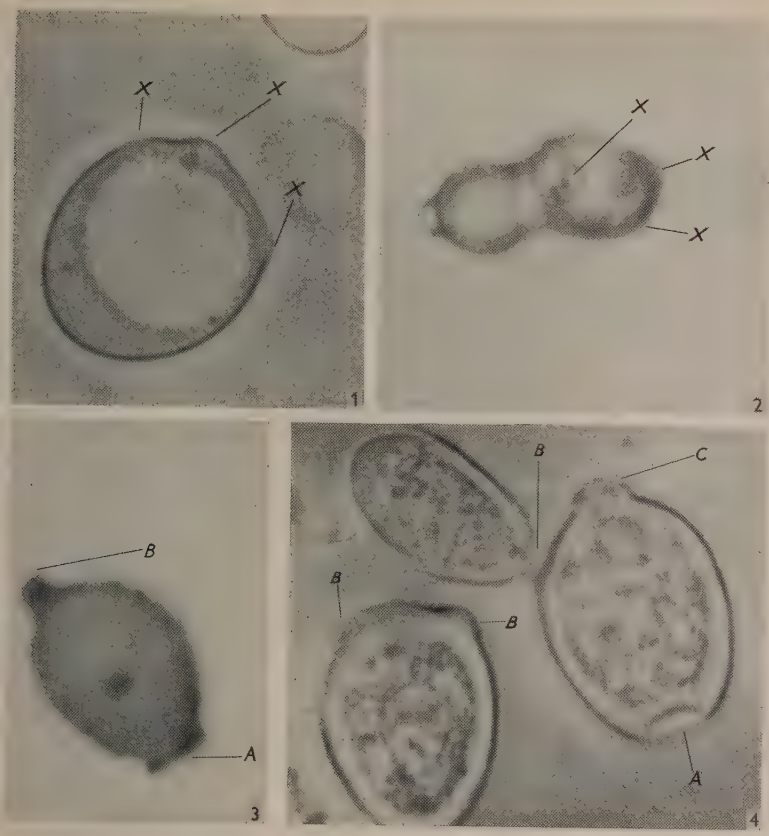
Fig. 2. Scars (marked *X*) on the cell wall of a cell growing on the surface of malt-agar. Wratten 38 A filter; × 1600.

Fig. 3. Birth scar (*A*) and bud scar (*B*) on the cell wall of a yeast cell. The bud scar has collapsed whilst the birth scar has remained intact. Newton crystal-violet method. Wratten 38 A filter; × 3600.

Fig. 4. Cells plasmolysed by heat. The birth scar (*A*) and first bud scar (*C*) are of a greater diameter than the other scars (*B*). Photographed using mercury violet illumination. Ilford 601 filter; × 3600.

Fig. 5. The positions of origin of buds one to eight. The birth scar (*A′*) is at the pole of the long axis of the cell; × 3500.

(*Received* 28 *May* 1949)



Figs. 1–4

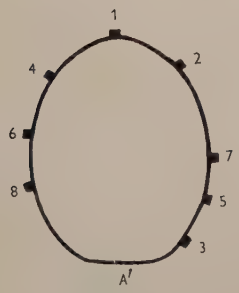


Fig. 5

A. A. Barton—Aspects of cell division in *S. cerevisiae*. Plate 1

The Reducing Sugars Liberated during the Bacterial Synthesis of Polysaccharides from Sucrose

By W. G. C. FORSYTH* and D. M. WEBLEY
Macaulay Institute for Soil Research, Aberdeen

SUMMARY: Paper chromatography was used for studying qualitatively and quantitatively the reducing sugars liberated from sucrose during the synthesis of polysaccharides by certain bacteria. Glucose and fructose were the only sugars detected, varying in amount with the bacteria and with the type of polysaccharide synthesized. The results suggest that both polymerizing and hydrolytic enzymes were present.

In a preliminary note (Forsyth & Webley, 1948) attention was drawn to the possible use of the method of paper chromatography of sugars (Partridge, 1946, 1948; Forsyth, 1948), especially when applied on a quantitative basis (Flood, Hirst & Jones, 1947), for studying the carbohydrate metabolism of micro-organisms. This paper deals in more detail with the application of this method to the study of the reducing sugars produced from sucrose during the synthesis of polysaccharides by certain bacteria.

CULTURES

The organisms we studied were the following: *Leuconostoc mesenteroides* (N.C.T.C. no. 3351) and an enzyme preparation from *L. mesenteroides* strain B as used by Hehre (1946); *Bacillus megatherium* (A.C.T.C. strain no. 697); *B. polymyxa* (a strain originally isolated in Prof. Kluvyer's laboratories) (Forsyth & Webley, 1949); *Rhizobium radicicolum*, no. 317; *Bacillus circulans* (strain no. 760, *B. Krzemieniewski*) smooth strain (Forsyth & Webley, 1949).

Each of these organisms is capable of synthesizing polysaccharides from sucrose in copious amounts. Most strains of *Leuconostoc mesenteroides* produce copious yields of a dextran from sucrose but not from glucose or fructose or any other sugar (Tarr & Hibbert, 1931). *Bacillus megatherium* synthesizes a levan from sucrose (Cooper & Preston, 1935). Although *B. megatherium* is also capable of synthesizing a polysaccharide of a different nature from monosaccharides it does not do this on the medium used here (Forsyth & Webley, 1949); only levan is formed. *B. polymyxa* synthesizes a levan from sucrose (Cooper, 1939). Forsyth & Webley (1949) have shown that besides levan another polysaccharide composed of glucose, mannose and uronic acid units is produced by this organism. *Rhizobium radicicolum* synthesizes a polysaccharide containing glucose with a small amount of uronic residues from sugars (Cooper, Daker & Stacey, 1938). *Bacillus circulans*, 760, synthesizes a glucose-mannose-uronic acid polymer from either sucrose or monosaccharides (Forsyth & Webley, 1949). It has been reported that reducing sugars are

* Present address: Colonial Microbiological Research Institute, Trinidad, British West Indies.

formed during the production of dextrans (Cooper & Carruthers, 1936) and levans (Genghof, Hehre & Neill, 1946), but no study appears to have been made on any sugars liberated during the synthesis of other polysaccharides.

METHODS

The organisms were grown in Tarr & Hibbert's (1931) liquid medium (unless otherwise stated) with the addition of 20 % yeast extract to give a final concentration of 2 % (v/v). The yeast extract was prepared by steaming a 20 % suspension (fresh weight) of baker's yeast for 1 hr., decanting and filtering through a Seitz filter-disk. The sucrose concentration was 10 % (w/v). A loopful of a 48 hr. culture of the required organism was inoculated into this medium (10 ml.) and the culture flask (50 ml.) incubated at 23°. The enzyme preparation from *Leuconostoc mesenteroides*, strain B, was prepared by the method of Hehre (1946). Three litres of the culture medium used by him were usually employed and the final precipitate taken up in 20 ml. 0·025 M citrate buffer (pH 6·3). Sucrose (10 %) in 0·1 M acetate buffer (pH 5·6) was used as substrate and the enzyme-substrate mixture was made up of 2 ml. of each solution, giving an initial concentration of 5 % sucrose. The enzyme-substrate mixture was incubated at 23° and the synthesized dextran precipitated by 2 volumes of ethanol.

The sugars were determined at any desired time during growth by the transfer of a definite volume of the liquid, 15 μl., on to a paper strip. This was initially done by means of a flame-sterilized platinum loop by weighing, but later a sterile micro-pipette was used. The micro-volume was spotted along a paper strip and the sugars chromatographed and qualitatively and quantitatively determined by the methods of Partridge (1946, 1948), Forsyth (1948), and Flood *et al.* (1947). An internal sugar standard was not used since it might have interfered with bacterial growth. Sucrose was detected on the paper with resorcinol (Forsyth, 1948) and estimated in the usual manner (Flood *et al.* 1947) after a preliminary hydrolysis with dilute oxalic acid. Sufficient concentrated oxalic acid solution was added to the sucrose extracted from the paper chromatogram to give a final concentration of 0·5 %. After heating on a boiling water-bath for 1 hr. the sucrose was estimated by Somogyi's (1945) reagent in the same manner as the other sugars, using a standard sucrose solution similarly treated. Samples of the polysaccharides produced were precipitated with ethanol, hydrolysed, and the hydrolysates examined by chromatography to confirm the nature of the polysaccharides.

RESULTS

Sucrose, glucose and fructose were the only sugars detected during the growth of all the polysaccharide-synthesizing bacteria. *L. mesenteroides* produced both glucose and fructose when grown on sucrose as shown in Table 1.

The amount of glucose was, however, very small compared with fructose. In view of this result it was thought of interest to try the dextran-synthesizing enzyme as prepared by Hehre (1946) from *L. mesenteroides*. Using 2 ml. of

enzyme preparation and 2 ml. of 10 % sucrose in 0·1 M acetate buffer the results shown in Table 2 were obtained.

It is clear from Table 2 that the enzyme preparation also liberated glucose as well as fructose, although in small amounts, during the synthesis of dextran. It will be seen from Table 3 that the levan-synthesizing *Bacillus megatherium* also produced glucose and fructose during its growth. As one might expect very much more glucose than fructose was liberated.

Table 1. *Utilization of sucrose by* Leuconostoc mesenteroides (N.C.T.C. *no.* 3351)

Grown in Tarr & Hibbert's (1931) liquid medium + 2 % yeast extract at 23°.

Period of incubation (days)	Sucrose present (%)	Fructose present (%)	Glucose present (%)
0	10·00	—	—
1	7·40	0·92	0·11
2	4·50	2·08	0·49
4	0·99	4·18	0·31
6	0·33	4·02	0·14
8	—	3·91	0·18
10	—	3·22	0·14

Table 2. *Utilization of sucrose by enzyme preparation from* Leuconostoc mesenteroides, *strain B, at* 23°

Period of incubation (hr.)	Weight of gum (g.)	Sucrose present (%)	Fructose present (%)	Glucose present (%)
0	—	5·00	—	—
24	0·0941	0·30	1·40	0·06
48	0·1046	0·39	1·65	0·07

Table 3. *Utilization of sucrose by* Bacillus megatherium, *strain no.* 697, *grown in Tarr & Hibbert's* (1931) *liquid medium with* 2 % *yeast extract at* 23°

Period of incubation (days)	Sucrose present (%)	Fructose present (%)	Glucose present (%)
0	10·0	—	—
2	3·5	0·66	2·34
4	1·04	0·72	3·08

Although *B. polymyxa* grew well and produced about 20 % of polysaccharide, only traces of glucose and fructose could be detected after 2 or 4 days' incubation at 23°. *Rhizobium radicicolum*, 317, was grown in a liquid medium of the following composition: K_2HPO_4, 0·05 %; $MgSO_4.7H_2O$, 0·02 %; NaCl, 0·02 %; $NaNO_3$, 0·1 %; yeast water, 2 %; and sucrose, 10 %. This organism would not grow satisfactorily in Tarr & Hibberts's medium. In the above liquid medium it grew well and produced copious yields of gum. However, as in the case of *Bacillus polymyxa* only traces of glucose and fructose could be detected in the medium. *B. circulans*, 760, also produced only small amounts of glucose and

fructose in about equal quantity (2 days, glucose, 0·05 %, fructose 0·06 %; 4 days, glucose 0·04 %, fructose 0·08 %), although copious amounts of gum were produced and over 50 % of the sucrose was utilized in 4 days.

DISCUSSION

Apart from the probable desmolytic reactions taking place it is of interest that the bacteria which produce large quantities of reducing sugars (*Leuconostoc mesenteroides* and *Bacillus megatherium*) are those which are capable of producing polysaccharide only from sucrose and not from the constituent monosaccharides. The other bacteria could use any monosaccharide liberated for the synthesis of more polysaccharide. *B. megatherium* can produce a polysaccharide from monosaccharides but not on Tarr & Hibberts's medium (Forsyth & Webley, 1949).

During the production of dextran by *Leuconostoc mesenteroides* large quantities of reducing sugars are formed and it has generally been assumed that the glucose part of the sucrose is polymerized to dextran with simultaneous liberation of the fructose moiety (Cooper & Carruthers, 1936). Considerable interest is centred on this organism because of Hehre & Sugg's (1942) enzymic synthesis of dextran. They considered that the formation of dextran and reducing sugars from sucrose by their enzyme preparation was consistent with the equation (Hehre, 1941):

$$\underset{\text{Sucrose}}{n\,C_{12}H_{22}O_{11}} \rightarrow \underset{\text{dextran}}{(C_6H_{10}O_5)_n} + \underset{\text{fructose}}{n\,C_6H_{12}O_6}$$

That glucose as well as fructose is produced, although in small amount, by both the bacteria and by the enzyme system, suggests the presence of a hydrolytic as well as a polymerizing enzyme. The dextran produced in our experiments was hydrolysed and shown by chromatographic analysis to be indeed a polymer of glucose units only. This was done to eliminate the possibility that some levan might be produced with concurrent liberation of glucose.

Working with an enzyme, levansucrase, prepared from the cells of *Aerobacter levanicum*, Hestrin & Avineri-Shapiro (1944) showed that both fructose and glucose were liberated during levan synthesis. To some extent by analogy with the equation of Hehre (1941) for dextran production, they minimized the part played by the hydrolytic enzyme which produced fructose in their system. Our showing that both dextran- and levan-producers liberate glucose and fructose suggests that the hydrolytic part of the enzyme systems may be of some importance. We confirmed that the polysaccharide produced by *B. megatherium* was composed entirely of fructose units.

The chromatographic method gives a more direct and sensitive approach to problems in this field than previous methods, since it enables the sugars to be detected, identified and estimated independently of each other at any desired time during the growth of the micro-organism.

We wish to thank: Dr Ruth E. Gordon for the culture of *Bacillus megatherium* and *B. circulans*; Dr Hehre for the *Leuconostoc mesenteroides* strain B subculture; and Dr Nutman for the culture of *Rhizobium radicicolum*, no. 317; used in this work.

REFERENCES

Cooper, E. A. (1939). Polysaccharide synthesis by the bacilli. *J. Soc. chem. Ind., Lond.*, **58**, T 229.

Cooper, E. A. & Carruthers, A. (1936). Enzyme formation and polysaccharide synthesis by bacteria. II. *Biochem. J.* **30**, 1001.

Cooper, E. A., Daker, W. D. & Stacey, M. (1938). Enzyme formation and polysaccharide synthesis by bacteria. III. Polysaccharides produced by 'nitrogen-fixing' organisms. *Biochem. J.* **32**, 1752.

Cooper, E. A. & Preston, J. F. (1935). Enzyme formation and polysaccharide synthesis by bacteria. *Biochem. J.* **29**, 2267.

Flood, A. E., Hirst, E. L. & Jones, J. K. N. (1947). Quantitative estimation of mixtures of sugars by the paper chromatogram method. *Nature, Lond.*, **160**, 86.

Forsyth, W. G. C. (1948). Colour reagents for the paper chromatography of sugars. *Nature, Lond.*, **161**, 239.

Forsyth, W. G. C. & Webley, D. M. (1948). A method for studying the carbohydrate metabolism of micro-organisms. *Nature, Lond.*, **162**, 150.

Forsyth, W. G. C. & Webley, D. M. (1949). Polysaccharides synthesized by aerobic mesophilic spore-forming bacteria. *Biochem. J.* **44**, 455.

Genghof, D. S., Hehre, E. J. & Neill, J. M. (1946). Serological reactions of levans formed from sucrose and raffinose by certain bacilli. *Proc. Soc. exp. Biol., N.Y.*, **61**, 339.

Hehre, E. J. (1941). Production from sucrose of a serologically reactive polysaccharide by a sterile bacterial extract. *Science*, **93**, 237.

Hehre, E. J. (1946). The enzymatic synthesis of dextran. *J. biol. Chem.* **163**, 221.

Hehre, E. J. & Sugg, G. (1942). Serologically reactive polysaccharides produced through the action of bacterial enzymes. I. Dextran of *Leuconostoc mesenteroides* from sucrose. *J. exp. Med.* **75**, 339.

Hestrin, S. & Avineri-Shapiro, S. (1944). The mechanism of polysaccharide production from sucrose. *Biochem. J.* **38**, 2.

Partridge, S. M. (1946). Application of the paper partition chromatogram to the qualitative analysis of reducing agars. *Nature, Lond.*, **158**, 270.

Partridge, S. M. (1948). Filter-paper partition chromatography of sugars. I. General description and application to the qualitative analyses of sugars in apple juice, egg white and foetal blood of sheep. *Biochem. J.* **42**, 238.

Somogyi, M. (1945). New reagent for the determination of sugars. *J. biol. Chem.* **160**, 61.

Tarr, A. & Hibbert, H. (1931). Studies on reactions relating to carbohydrate polysaccharides. XXXVII. The formation of dextran by *Leuconostoc mesenteroides*. *Canad. J. Res.* **5**, 414.

(*Received* 11 *June* 1949)

Classification of the Streptococci of Subacute Bacterial Endocarditis

By J. S. PORTERFIELD
Department of Bacteriology, Liverpool University

SUMMARY: Twenty-nine strains of streptococci from patients with subacute bacterial endocarditis, three type cultures of *Streptococcus sanguis*, and eighty strains of streptococci from the blood of persons after dental extraction were tested physiologically and serologically.

Six endocarditis strains fall into Lancefield group H and four into Lancefield group D; the remaining nineteen were serologically heterogenous. Thirteen of the endocarditis strains produced a serologically reactive dextran in sucrose broth. Of these five were group H streptococci; the rest represented several serological types.

Of the three dextran-producing strains of *Str. sanguis* examined, types I and I/II were placed in Lancefield group H, whereas type II was serologically distinct.

The eighty strains from dental bacteraemias included ten which produced a dextran; five of these were group H streptococci.

The classification of the 'viridans' streptococci is notoriously unsatisfactory. Serological examination of strains from subacute bacterial endocarditis has revealed occasional 'viridans' streptococci which have been placed in the various Lancefield groups (Solowey, 1942; Wheeler & Foley, 1945; Penistan, 1945; Loewe & Alture-Werber, 1946). Hehre & Neill (1946) demonstrated that twenty-two out of forty-eight endocarditis streptococci synthesized a polysaccharide from sucrose broth, a property also possessed by nine haemolytic group H streptococci tested. The polysaccharide was identified chemically and serologically as a dextran, and it was noted that it reacted in high dilution with type II pneumococcus serum. Three of the twenty-two dextran-producing endocarditis strains had been submitted to these workers because they had reacted in precipitin tests with group H serum, but the only other dextran-producing strain tested did not react with this serum. Although there were many physiological similarities between the haemolytic group H streptococci and the polysaccharide-producing endocarditis strains, the writers pointed out that this did not necessarily mean that the latter should be classed as group H in the Lancefield system.

At about the same time another group of workers noted that about one-third of the streptococci from subacute bacterial endocarditis formed a homogenous variety or species which they termed *Streptococcus s.b.e.* (Loewe, 1945; Loewe & Alture-Werber, 1946; Loewe, Plummer, Niven & Sherman, 1946). Niven, Kiziuta & White (1946) described polysaccharide formation in sucrose broth as an outstanding feature of these strains, and considered it highly probable that the dextran-producing streptococci described by Hehre & Neill were identical with the species they were studying. White & Niven (1946) gave a detailed account of the other physiological properties of this species, based upon an examination of forty-two cultures, and recommended the alternative name of *Str. sanguis*. Washburn, White & Niven (1946), placed thirty-two

strains of *Str. sanguis* in type I, five in type II and five in type I/II, using precipitin tests with sera prepared against heat-killed organisms. These type-specific sera did not react with Lancefield extracts of groups A to G, but apparently no group H extract was tested. All forty-two strains of *Str. sanguis* reacted with I/II serum, although the five type II extracts gave only weak precipitation. It was felt that type I/II strains possessed two type-specific antigens rather than a group antigen.

Dodd (1949) examined types I and II of *Str. sanguis*, and stated that extracts of both reacted with group H serum. According to her tables, however, type I extract reacted with type II serum, and vice versa. Dodd claimed, by using absorbed sera, to be able to recognize five precipitin types within group H, but it is questionable whether absorption had been adequate.

MATERIALS AND METHODS

Sources of strains and sera. Of twenty-nine endocarditis streptococci ten were kindly supplied by Prof. L. P. Garrod, nine were isolated by Dr M. G. McEntegart working in this department, and ten by the writer. Nine haemolytic streptococci of Lancefield group H were kindly supplied by Dr Fry, who also provided two samples of group H serum.

One haemolytic streptococcus of Lancefield group H was received from Dr V. D. Allison, who also confirmed that another haemolytic strain (63A from a dental bacteraemia) was a member of group H.

Str. sanguis, types I, II and I/II, were obtained from the National Collection of Type Cultures (N.C.T.C. nos. 7863, 7864 and 7865 respectively).

Eighty different streptococci were isolated from cultures of the blood from 61 of 100 normal persons sampled after dental extractions (McEntegart & Porterfield, 1949).

Stock cultures were maintained on horse-blood agar slopes at 4°. Sera prepared against streptococci of Lancefield groups A to K, excluding E, and pneumococcus type II, were obtained from Burroughs, Wellcome and Co. Ltd.

Cultural and biochemical examinations. Fermentation tests were carried out in 1 % (w/v) solutions of glucose, mannitol, lactose, salicin, sucrose, inulin and raffinose in peptone water, to which was added 5 % (v/v) horse serum and Andrade's indicator. Acid production was recorded after 48 hr. incubation at 37°. The effect on litmus milk was also noted.

Hydrolysis of arginine was determined in the following medium, slightly modified from Niven, Smiley & Sherman (1942). (Quantities here and elsewhere in per cent w/v unless otherwise mentioned.) Peptone, 0·5; Marmite, 0·5; K_2HPO_4, 0·2; glucose, 0·5; D-arginine monohydrochloride, 0·3; pH 7·0. Readings were made after 3 days' incubation at 37° by adding one drop of Nessler's reagent to one large loopful of supernatant fluid on a porcelain tile. A heavy orange-coloured precipitate was interpreted as a positive reaction. Results were confirmed by adding 0·25 ml. of Nessler's reagent to the test medium after 7 days' incubation.

Growth at 45° was determined in 1 % glucose-peptone water containing 5 % (v/v) horse serum and Andrade's indicator. Tubes were heated to 45° and

inoculated with 0·05 ml. of an 18 hr. broth culture. Growth was detected by a change in the indicator, readings being made after 24 and 48 hr. incubation. Growth in broth containing 6·5 % NaCl was detected by the presence of visible turbidity after 48 hr. incubation at 37°.

Haemolysis was observed on blood-agar plates containing 5 % (v/v) horse blood incubated aerobically and anaerobically.

Growth on bile-blood agar: plates containing 5 % (v/v) horse blood with 10 or 40 % (v/v) ox bile were streaked with the strain to be tested and incubated at 37°. Bile-tolerant strains produced clearly visible colonies after 48 hr.

The colonial appearance was studied on 5 % sucrose agar plates incubated aerobically and anaerobically. The medium employed was a slight modification of that suggested by Niven, Smiley & Sherman (1941) and had the following composition: peptone 1·0 %; Marmite, 1·0 %; K_2HPO_4, 0·3 %; gelatin, 5·0 %; agar, 1·5 %; sucrose, 5·0 %.

Dextran-production in 5 % sucrose broth was tested in the following medium, adopted from Niven, *et al.* (1946): peptone, 1·0 %; Marmite, 0·5 %; K_2HPO_4, 0·5 %; sucrose, 5·0 %; pH 7·4. After 24 or 48 hr. incubation certain strains considerably increased the viscosity of this medium, which occasionally set solid. This property was recorded as 'visible gel formation in 5 % sucrose'. One large loopful of the supernatant fluid of a 48 hr. culture was mixed with 0·05 ml. of 0·85 % NaCl on a porcelain tile giving a dilution of approximately 1/20. The mixture was then layered on top of 0·05 ml. of type II pneumococcus antiserum in a small precipitin tube, and the development of a ring precipitate at the interface was taken as a positive reaction and was recorded as 'dextran production in 5 % sucrose broth'. Preliminary tests indicated that the supernatant fluid was highly acid and gave a precipitate with normal serum, but this effect was abolished by diluting the fluid 1/10 or more. No attempt was made to determine the quantity of dextran produced, but certain strains gave a positive reaction when the supernatant fluid was diluted 1/320 or 1/640. Strains which were negative after 48 hr. incubation invariably remained negative when tested at intervals throughout 1 week.

Serological tests. Antisera were prepared in rabbits using formol-killed suspensions according to the method of Lancefield (1938*a*). Acid extracts of streptococci for precipitin tests were prepared by Lancefield's (1933) method. Absorption tests were carried out using a two-stage modification of Lancefield's technique (Lancefield, 1938*b*).

RESULTS

Dextran was produced in sucrose broth by nine of eleven haemolytic group H streptococci, by all three strains of *Str. sanguis*, by thirteen of twenty-nine endocarditis strains and by ten of eighty streptococci recovered from dental bacteraemias. A strong precipitin reaction with group H serum was noted with Lancefield extracts of all the haemolytic group H strains, with types I and I/II of *Str. sanguis*, with six endocarditis and five bacteraemia strains. The relationship between dextran production and reaction with group H serum is shown in Table 1.

For convenience, all those non-haemolytic streptococci giving a positive precipitin test with group H serum will be referred to as class X strains, and the remainder as class Y strains, irrespective of source, dextran-production, or other physiological properties. The corresponding antisera will be referred to as class X and class Y sera.

Table 1. *Relationship between dextran-production and reaction of Lancefield extracts of various streptococci with group H serum*

Source of strains	No. examined	Dextran production	No. reacting with group H serum
Haemolytic group H	11	Positive 9	9
		Negative 2	2
Endocarditis	29	Positive 13	5
		Negative 16	1
Str. sanguis	3	Positive 3	2
		Negative 0	0
Dental bacteraemia	80	Positive 10	5*
		Negative 70	0

* One dextran-producing bacteraemia strain was haemolytic and is also included in the eleven haemolytic group H strains.

Six class X sera were prepared against four endocarditis and two bacteraemia streptococci. Three endocarditis strains and both bacteraemia strains produced a dextran.

Fifteen class Y sera were prepared against nine endocarditis strains (three of which produced dextran) *Str. sanguis*, type II, two unclassified bacteraemia strains, and three strains which gave mucoid colonies after 24 hr. aerobic incubation on sucrose agar, and otherwise corresponded to the description of *Str. salivarius* (Sherman, Niven & Smiley, 1943). One of these last strains came from the dental bacteraemia series, in which it was the sole representative of its species; the other two strains came from normal mouths.

Lancefield extracts of the haemolytic group H streptococci, of the endocarditis, of the *Str. sanguis* strains, and of the ten dextran-producing streptococci in the bacteraemia series were tested against all the class X and class Y sera. Extracts of all the class X strains reacted with each of the six class X sera, and some also reacted with a serum prepared against one of the strains of *Str. salivarius* in class Y, but with no other class Y serum. With extracts prepared against the class Y strains the results were very different, and only one serum in class Y (apart from the anti-salivarius serum already mentioned) reacted definitely with any but the homologous extract. The exception, a serum prepared against a dextran-producing streptococcus 'Robinson', reacted with three other endocarditis strains and three bacteraemia strains, all of which were dextran-producers which failed to react with group H serum. These facts are summarized in Table 2.

Since all twelve strains in class X reacted with three different group H sera, and since all eleven haemolytic group H strains reacted with six antisera prepared against class X strains, when tested by the precipitin technique with

Lancefield extracts, it seemed highly probable that the strains in class X were non-haemolytic varieties of Lancefield group H. Since, however, a sharing of antigens might have been responsible, absorption tests were undertaken, using a Burroughs Wellcome group H serum, and the haemolytic group H 'Challis' strain originally isolated by Hare. A bacteraemia strain 84A, and its corresponding serum were selected to represent class X, since this was the most

Table 2. *Relationships between haemolytic group H streptococci and certain non-haemolytic streptococci*

Lancefield extracts of streptococci	Reaction with three group H sera	Reaction with six class X sera	Reaction with fifteen class Y sera
11 haemolytic group H	All positive	All positive	All negative*
12 non-haemolytic class X	All positive	All positive	All negative*
29 non-haemolytic class Y	All negative	All negative	Homologous reaction only†

* Certain haemolytic and class X strains reacted with one serum in class Y.
† Another class Y serum ('Robinson') reacted with six strains in class Y as well as the homologous strain.

Table 3. *Cross-absorption tests with group H (Challis) antiserum and a 'viridans' streptococcus 84A antiserum*

Lancefield extracts of strains	Group H serum unabsorbed	Group H serum absorbed with cells of: Challis H	84A	Group F
Challis (group H)	+++	–	–	+++
84A	+++	–	–	+++
Crompton	+++	–	–	+++
332	+++	–	–	+++

Lancefield extracts of strains	84A serum unabsorbed	84A serum absorbed with cells of: Challis H	84A	Group F
Challis (group H)	+++	–	–	+++
84A	+++	++	–	+++
Crompton	+++	–	–	+++
332	+++	–	–	+++

potent serum, and also the most plentiful. Extracts of two other class X strains, Crompton and 332, were included in the tests. The results of cross-absorption tests using these sera and strains, and a heterologous group F strain are shown in Table 3.

Both 84A and 'Challis' cells completely absorbed group H antibody, whereas group F cells left the reaction unchanged. Serum 84A was completely absorbed by 84A cells, unaffected by group F cells, and partially absorbed with 'Challis' cells, so that the resultant serum reacted with an extract of 84A, but not with a group H extract, nor with extracts of the other class X strains. The group H cells removed all the group-specific antibody, but left some type-specific antibody which still reacted with the homologous extract, but not with any other extracts.

All the other streptococci in class X except one absorbed group H antibody completely. The exception, an endocarditis strain, diminished the reaction considerably. No strains of class Y, included as controls, absorbed group H antibody (Table 4).

Table 4. *Absorption of group H antiserum by cells of various 'viridans' streptococci*

Absorbing strain	Source	Class	Reaction with Lancefield extract of Challis H	Eliffe	332
Cohen	Endocarditis	X	–	–	–
Crompton	,,	X	–	–	–
Gardner	,,	X	–	–	–
Eliffe	,,	X	–	–	–
Moss	,,	X	–	–	–
Bates	,,	X	–	+	+
Cooper	,,	Y	+++	+++	+++
Fairhurst	,,	Y	+++	+++	+++
Sampson	,,	Y	+++	+++	+++
Robinson	,,	Y	+++	+++	+++
Sanguis, type I	,,	X	–	–	–
Sanguis, type I/II	,,	X	–	–	–
Sanguis, type II	,,	Y	+++	+++	+++
31 A	Bacteraemia	X	–	–	–
332	,,	X	–	–	–
74 A	,,	X	–	–	–
84 A	,,	X			
Group F	Control	—	+++	+++	+++
Unabsorbed	Control	—	+++	+++	+++

A comparison of the physiological properties of eleven haemolytic group H streptococci and twelve class X non-haemolytic streptococci is shown in Table 5. Apart from the single characteristic of haemolysis, there is a high degree of correlation between the two collections of strains, and variations between the groups are no more than those within them. In view of the weight of serological and physiological evidence, there can be little doubt that the strains which have been placed together as class X are non-haemolytic or 'viridans' varieties of Lancefield group H.

An attempt was made to see whether, by altering the conditions of growth, the 'viridans' strains of group H could be induced to give β haemolysis. Strains were incubated on 5 % (v/v) horse-, sheep- and rabbit-blood agar plates under aerobic and anaerobic conditions, and representative strains were grown in deep-blood agar plates (Brown, 1919) using blood from the various species. Two strains were carried through twelve transplants on anaerobic horse-blood agar plates, but in no case did any strain produce β haemolytic colonies.

The classification of the remaining endocarditis strains is of some interest. One strain was a typical *Str. faecalis*, which fermented glucose, mannitol, lactose, salicin and sucrose, but not inulin or raffinose, brought about rapid and complete reduction of litmus milk, hydrolysed arginine, grew at 45°, in the presence of 6·5 % NaCl, on 10 and 40 % bile-blood agar, and survived heating to 60° for 30 min. Three other strains had several characteristics of enterococci, in that they brought about a rapid and complete reduction of

litmus milk, and were salt and bile tolerant; but they failed to ferment mannitol or hydrolyse arginine, did not grow at 45° and were not heat resistant. Moreover, these strains were 'viridans' streptococci, whereas the first was completely non-haemolytic. Lancefield extracts of the *Str. faecalis* strain reacted strongly with group D serum. Crude extracts of the other three strains did not react,

Table 5. *Comparison of the physiological properties of* 11 *haemolytic group H streptococci and* 12 *selected 'viridans' streptococci (class X)*

	No. showing designated characteristic	
	11 group H	12 class X
β haemolysis 5 % horse-blood agar anaerobically	11	0
Fermentation of:		
Glucose	11	12
Mannitol	0	0
Lactose	10	9
Salicin	11	12
Sucrose	11	12
Inulin	11	12
Raffinose	1	3
Acid and clot in litmus milk	8	7
Hydrolysis of arginine	11	11
Growth at 45°	11	4
Growth in presence of 6·5 % NaCl	0	0
Growth on 10 % bile-blood agar	11	11
Growth on 40 % bile-blood agar	8	10
Visible gel in sucrose broth	3	11
Dextran-production in sucrose broth	9	11

but extracts concentrated by ethanol gave a faint reaction. Possibly these were representatives of *Str. bovis*, although they had several differences from the characteristics of that type as described by Sherman & Stark (1931) and by Shattock (1949).

The remaining endocarditis strains could not be placed in any of Lancefield groups A–K (excluding E). Four fell into the serological type 'Robinson' and, with three bacteraemia strains, were able to absorb precipitin from a serum prepared against the strain 'Robinson'. The physiological properties of these seven strains were similar to those of *Str. sanguis*, type II, all of them producing a dextran in sucrose broth and all failing to ferment inulin, to hydrolyse arginine or to grow on bile-blood agar. However, the two types were serologically distinct.

The development of mucoid colonies after 3–5 days' anaerobic incubation on sucrose agar was correlated with the property of dextran-production in sucrose broth. Parallel aerobic cultures of these strains showed only slight glassiness of the colonies. No endocarditis strain produced large mucoid colonies after 24 hr. aerobic incubation on sucrose agar or could otherwise be identified as *Str. salivarius*, and only one such strain was found in the bacteraemia series. The classification of the bacteraemia streptococci is given in Table 6.

Table 6. *Classification of eighty streptococci from dental bacteraemias*

Classification	β haemolytic	No. of strains
Group F	+	1
Group G	+	1
Group H	+	1
	−	4
Group K	+	1
	−	1
Str. salivarius	−	1
Unclassified ? *Str. mitis*	−	71

DISCUSSION

This investigation demonstrates that approximately 45 % of streptococci from cases of subacute bacterial endocarditis produced a serologically reactive dextran in sucrose broth. This property was less common among other streptococci, being found in only ten out of eighty streptococci from dental bacteraemias, but was the rule in all group H strains. Neill, Sugg, Hehre & Jaffe (1941) tested seventy-eight streptococci of various Lancefield groups and found that only group H included dextran-producing strains, and the writer, in a small unreported series, had the same experience.

A proportion of the dextran-producing 'viridans' streptococci, including types I and I/II of *Str. sanguis*, were found to belong to Lancefield group H, and the physiological properties of these strains agreed very closely with those given by White & Niven (1946) for that species. However, exceptions were noted. A strain might be atypical in one or more of the physiological tests, and still fall into Lancefield group H. Neither hydrolysis of arginine, dextran-production, bile-tolerance, nor fermentation of inulin without fermentation of raffinose could be accepted as absolute criteria, although they were shown by the majority of group H strains, whether haemolytic or 'viridans'.

The remaining dextran-producing streptococci fell into at least four different serological types. No strains were found to correspond to type II of *Str. sanguis*, nor did it appear, as Dodd (1949) contended, that type II is in any way related to Lancefield group H.

Loewe suggested that although strains of *Str. sanguis* were sensitive to penicillin *in vitro*, patients infected with these organisms responded poorly to therapy, the relapse rate being higher, and the proportion of successful results lower, than with all other types of streptococcal endocarditis (Loewe, 1945; Loewe & Alture-Werber, 1946). Priest, Smith & McGee (1947) support this suggestion. There were four patients who relapsed in the twenty-nine studied by the writer. Two were treated in 1945 with small doses of penicillin and both responded well to a second larger course. The two others were infected with group H streptococci. One was cured by 2 mega units daily for 6 weeks, the other relapsed a second time after ½ mega unit daily for 4 weeks, but was cured by a third course of 1 mega unit of penicillin daily for 6 weeks. A third patient with a group H infection failed to respond until the dosage of penicillin was raised to 16 mega units daily, although the organism was only twice as resistant as the Oxford staphylococcus. This patient eventually gave sterile

blood cultures after 480 mega units administered over 50 days, but died of cardiac failure 10 days later. Although this may be taken as confirming the refractory nature of some infections with group H streptococci, evidence based upon such small numbers can hardly be considered satisfactory.

Even if only one-half of the dextran-producing streptococci described by White & Niven (1946) and by Hehre & Neill (1946) be accepted as group H strains, it would appear that a significant proportion of cases of subacute bacterial endocarditis are due to this group. Precise serological classification of the infecting strain facilitates search for the primary focus and may be useful in distinguishing between a relapse and a reinfection with a different organism. White & Niven (1946) failed to find *Str. sanguis* in the normal mouth and throat, although haemolytic group H streptococci are not uncommon there (Hare, 1935). The present investigation showed that group H streptococci entered the blood stream after dental extractions and indicated one possible source, though not necessarily the only source, of these organisms in subacute bacterial endocarditis.

The author wishes to thank Dr V. D. Allison, Dr R. M. Fry and Prof. L. P. Garrod for many of the strains examined in this work.

REFERENCES

Brown, J. H. (1919). The use of blood agar for the study of streptococci. *Monogr. Rockefeller Inst. med. Res.* no. 9. New York.

Dodd, R. L. (1949). Serologic relationship between streptococcus group H and *Streptococcus sanguis*. *Proc. Soc. exp. Biol., N.Y.*, **70**, 598.

Hare, R. (1935). The classification of haemolytic streptococci from the nose and throat of normal human beings by means of precipitin and biochemical tests. *J. Path. Bact.* **41**, 499.

Hehre, E. J. & Neill, J. M. (1946). Formation of serologically reactive dextrans by streptococci from subacute bacterial endocarditis. *J. exp. Med.* **83**, 147.

Lancefield, R. C. (1933). A serological differentiation of human and other groups of haemolytic streptococci. *J. exp. Med.* **57**, 571.

Lancefield, R. C. (1938*a*). A micro precipitin-technique for classifying haemolytic streptococci and improved methods for producing antisera. *Proc. Soc. exp. Biol., N.Y.*, **38**, 473.

Lancefield, R. C. (1938*b*). Two serological types of group B haemolytic streptococci with related, but not identical, type-specific substances. *J. exp. Med.* **67**, 25.

Loewe, L. (1945). The combined use of penicillin and heparin in the treatment of subacute bacterial endocarditis. *J. Canad. med. Ass.* **52**, 1.

Loewe, L. & Alture-Werber, E. (1946). The clinical manifestations of subacute bacterial endocarditis caused by *Streptococcus s.b.e.* *Amer. J. Med.* **1**, 353.

Loewe, L., Plummer, N., Niven, C. F. & Sherman, J. M. (1946). Streptococcus s.b.e. in subacute bacterial endocarditis. *J. Amer. med. Ass.* **130**, 257.

McEntegart, M. G. & Porterfield, J. S. (1949). Bacteraemia following dental extractions. *Lancet*, ii, 596.

Neill, J. M., Sugg, J. Y., Hehre, E. J. & Jaffe, E. (1941). Influence of sucrose upon production of serologically reactive material by certain streptococci. *Proc. Soc. exp. Biol., N.Y.*, **47**, 339.

Niven, C. F., Kiziuta, Z. & White, J. C. (1946). Synthesis of a polysaccharide from sucrose by *Streptococcus s.b.e.* *J. Bact.* **51**, 711.

NIVEN, C. F., SMILEY, K. L. & SHERMAN, J. M. (1941). The production of large amounts of a polysaccharide by *Streptococcus salivarius*. *J. Bact.* **41**, 479.

NIVEN, C. F., SMILEY, K. L. & SHERMAN, J. M. (1942). The hydrolysis of arginine by streptococci. *J. Bact.* **43**, 651.

PENISTAN, J. L. (1945). A non-haemolytic group K streptococcus from a case of endocarditis. *J. Path. Bact.* **57**, 123.

PRIEST, W. S., SMITH, J. M. & MCGEE, C. J. (1947). Penicillin therapy of subacute bacterial endocarditis. *Arch. intern. Med.* **79**, 333.

SHATTOCK, P. M. F. (1949). The streptococci of group D: the serological grouping of *Streptococcus bovis* and observations on serologically refractory group D strains. *J. gen. Microbiol.* **3**, 80.

SHERMAN, J. M., NIVEN, C. F. & SMILEY, K. L. (1943). *Streptococcus salivarius* and other non-haemolytic streptococci of the human throat. *J. Bact.* **45**, 249.

SHERMAN, J. M. & STARK, P. (1931). Streptococci which grow at high temperatures. *J. Bact.* **22**, 275.

SOLOWEY, M. (1942). A serological classification of viridans streptococci with special reference to those isolated from subacute bacterial endocarditis. *J. exp. Med.* **76**, 109.

WASHBURN, M. R., WHITE, J. C. & NIVEN, C. F. (1946). *Streptococcus s.b.e.*: immunological characteristics. *J. Bact.* **51**, 723.

WHEELER, S. M. & FOLEY, G. E. (1945). A note on the serologic classification of streptococci isolated from subacute bacterial endocarditis. *Amer. Heart J.* **30**, 511.

WHITE, J. C. & NIVEN, C. F. (1946). A streptococcus associated with subacute bacterial endocarditis. *J. Bact.* **51**, 717.

(*Received* 24 *June* 1949)

The Effect of the Gas-Phase on Differential Inhibition of Intestinal Bacilli

By J. BRODIE and W. SHEPHERD
Department of Bacteriology, University of St Andrews, Medical School, Dundee

SUMMARY: By adjusting the electrolyte content of variously compounded bile-salt media, the growth of coliform bacilli and R forms of intestinal pathogens can be suppressed leaving the S forms of the latter unaffected. Anaerobiosis abolishes the inhibitory effect whereas increased oxygen concentrations enhance it. The degrees of inhibition depend upon the balance of bile salt, electrolyte and oxygen concentration.

Previous experiments (Brodie, 1948) showed that differential inhibitory effects among intestinal bacilli could be obtained when the organisms were grown aerobically in certain media, upon varying the bile salt:electrolyte ratio. The syneretic quality of the gelling agents affected the end result. Similar differential effects with similarly compounded media were obtained in shallow-layer fluid cultures, including fluid gelatin-containing media at 37° (Brodie & Shepherd, 1949). With the above media, the organisms most resistant to inhibition are the smooth forms of intestinal pathogens. The use of a shallow layer of fluid medium based upon these findings gave an improved isolation rate of intestinal pathogens from morbid material (Brodie, 1949).

It was observed (Brodie & Shepherd, 1949) that in shake cultures, the higher the concentration of the electrolyte the less tolerant of air the organisms became, with the exception of the smooth form of the *Sonne* III bacillus; moreover, the lethal effect of bile salt + electrolyte fluid media on the R and S forms of that organism was influenced more markedly by the depth of the layer of medium rather than by the duration of the exposure to the media. In view of these observations, it was decided to investigate the effects of the gas-phase upon the patterns of differential inhibition.

MATERIALS AND METHODS

All media were prepared and inoculated as described by Brodie (1948). The following organisms were studied: C1, *Bacterium coli communis* from human faeces; C2, *Bacterium coli communior* from human faeces; A1, *Aerobacter* sp. from milk; A2, *Aerobacter* sp. from water; SR, a rough form of a recently isolated *Sonne* III bacillus; SS, a smooth form of the same micro-organism; R and S forms of Flexner bacilli type V (N.C.T.C. 4832), type W (N.C.T.C. 4833), type X (N.C.T.C. 4834), type Y (N.C.T.C. 4836) and type Z (N.C.T.C. 4835); R and S forms of a fresh strain of the *Sonne* III bacillus. Of these organisms, C1, C2, A1, A2, SR and SS were the strains used previously (Brodie, 1948; Brodie & Shepherd, 1949).

The gases employed were hydrogen and oxygen, both of commercial standard,

and specially prepared nitrogen which contained less than 5 p.p.m. of carbon dioxide, no oxygen and no hydrogen (British Oxygen Co.).

Inoculated plates were exposed to the gases in a McIntosh & Fildes type anaerobic jar (no. 128B, Baskerville & Lindsay, Manchester). As much air as possible was removed from the jar by a Hyvac oil pump and then filled with the necessary gas; this procedure was repeated six times. When using hydrogen, the last traces of oxygen were burned out in the usual way. In preparing gas mixtures, the jar was filled with the principal gas, the necessary volume was evacuated, as recorded by a mercury manometer, and replaced with the subsidiary gas; a final adjustment of internal pressure was then made before incubation at 37° for 48 hr.

In the experiments with gas mixtures it was found necessary to prepare the mixtures immediately and to make no allowance for a rise in pressure on incubation. Adjustment to atmospheric pressure was made at room temperature immediately after mixing. Only by attention to these details were consistent results obtained.

RESULTS

Aerobic experiments

Initial experiments showed that when the R and S forms of the Flexner bacilli were grown aerobically on the surface of 2 % gels made with fibre agar (Thos. Morson and Son, London) containing quarter-strength peptic digest of serum, sodium chloride (0·5 % w/v) and taurocholate (0·5 % w/v) in the presence of 0·1 M sodium citrate (medium A), and 0·2 M sodium citrate (medium B), both R and S forms grew on A but only the S forms on B. The R and S forms of the new *Sonne* III bacillus behaved in the same way. This identity of behaviour by typical representative organisms of the dysentery group suggests that the mechanism of inhibition is common to the group.

The electron-microscope studies already made of strains SR, SS, C1, C2, A1 and A2 (Brodie & Shepherd, 1949) were extended to the new strains of Flexner and *Sonne* III bacilli. The R and S forms all behaved in the same way as the R and S forms of the original *Sonne* III bacillus, i.e. the S forms were unaffected by the higher concentrations of electrolytes in the presence of 0·5 % (w/v) taurocholate whereas the R forms similarly exposed showed plasmoptysis.

Variation of the gas-phase

Employing the same agar media as in the aerobic experiments, the effects of different gases, alone and mixed, were examined using the complete range of organisms.

In hydrogen and nitrogen, the coliform bacilli and all the R and S forms grew on media A and B. In 20 % (v/v) oxygen in nitrogen, the results were the same as obtained under aerobic conditions and of the four coliform bacilli A1 and A2 grew on medium A only.

When the oxygen content in nitrogen was increased, the differential properties of both media were enhanced, medium A in 40 % (v/v) oxygen in nitrogen giving results similar to those obtained on medium B in air or 20 %

(v/v) oxygen in nitrogen. In 60 % (v/v) oxygen in nitrogen medium B was completely inhibitory and medium A behaved as medium B in air or in 20 % (v/v) oxygen in nitrogen. To eliminate the possibility that increased oxygen tension alone was toxic, all the organisms were grown on nutrient agar and nutrient agar with 0·5 % (w/v) taurocholate under the same experimental conditions and in all instances good growth was obtained.

The effect of the agar gel

Throughout this work, the R and S forms were checked serologically and culturally. During one of these routine checks, a new batch of MacConkey's (1908) bile-salt medium was prepared substituting, as was customary during these investigations, peptically digested sheep's serum for the usual peptone. On this occasion, the agar was changed from fibre agar to Davis agar (Davis Gelatin Co., Christchurch, New Zealand). The alteration in brand of agar gave rise to a dramatic change in colony character of the R forms. Whereas on the fibre-agar medium these had given typically rough colonies, they now gave, after 24 hr. at 37°, colonies which were typically smooth. Picked from Davis agar medium and plated back on the fibre-agar bile-salt medium, the R forms now grew with their former typically rough colonies. Serial subculture of R forms on the fibre-agar medium always produced rough colonies; the same procedure on Davis agar medium always produced smooth colonies. Transfer of the R forms from fibre to Davis agar medium yielded smooth colonies and the reverse transfer yielded rough colonies. This alteration of colony type of the R forms on the Davis agar medium was not, however, attended by alteration in the serological character of the R forms.

CONCLUSIONS

From these and previous observations (Brodie, 1948; Brodie & Shepherd, 1949), it appears that the differential inhibition of R and S forms of dysentery bacilli and certain non-pathogenic intestinal bacilli in fluid and on solid media depends on the following factors.

(*a*) *In fluid media*, the depth of the layer, the balance between the bile-salt content and the concentration of the selected electrolyte and the availability of oxygen are the important factors. The consideration governing the selection of the electrolyte is that it must be one of the combinations of an anion and a cation in the ionic series of Hofmeister, the concentration to be used being directly related to its position in the series;

(*b*) *On solid media* the above criteria still apply but are contingent on the gel exhibiting a degree of syneresis sufficient to produce the equivalent of a shallow layer of the corresponding fluid medium on the surface of the gel.

The authors wish to thank Prof. W. J. Tulloch for advice and criticism and Prof. G. D. Preston of the Physics Department for permission to use the electron-microscope on loan from the Department of Scientific and Industrial Research. This paper forms part of the research work undertaken by one of us (J. Brodie) under the terms of the Ernest Hart Memorial Scholarship awarded by the British Medical Association.

REFERENCES

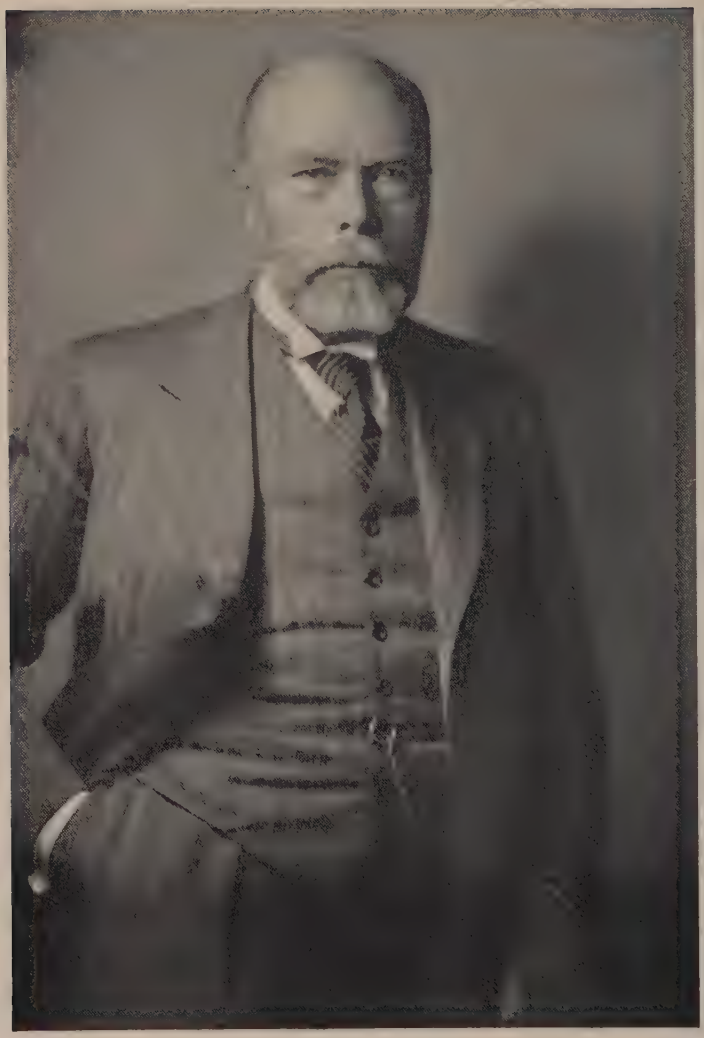
Brodie, J. (1948). Observations on the differential inhibition of coliform bacilli and rough variants of intestinal pathogens. *J. gen. Microbiol.* **2**, 1.

Brodie, J. (1949). Shallow-layer fluid enrichment method for intestinal pathogens. *J. Path. Bact.* **61**, 120.

Brodie, J. & Shepherd, W. (1949). Further observations on the differential inhibition of coliform bacilli and rough variants of intestinal pathogens. *J. gen. Microbiol.* **3**, 74.

MacConkey, A. T. (1908). Bile-salt media and their advantages in some bacteriological examinations. *J. Hyg., Camb.*, **8**, 322.

(*Received* 27 *June* 1949)



S. ORLA-JENSEN (1870–1949)

Honorary Member of the Society for General Microbiology

No. 1, Volume 4 of the Journal of General Microbiology was issued on 24 January 1950

Obituary Notice

S. ORLA-JENSEN

26 November 1870–24 June 1949

Professor, Dr.phil. et scient., S. Orla-Jensen died on 24 June 1949, at the age of seventy-eight. Microbiologists throughout the world will regret this loss to the international science of microbiology to which he had devoted all his efforts for fifty years, and which is so greatly indebted to this pathfinder in the bacterial world.

Orla-Jensen was educated as a chemical engineer, but very soon his interests were directed towards the young science of microbiology and fermentation physiology. He spent two years in the brewing industry but soon developed an interest in dairy bacteriology which was at that time rapidly increasing in scope, with many unsolved problems arising. Orla-Jensen exerted himself in the study of this field of microbiology throughout the rest of his life. He had the good fortune to study with some of the outstanding workers in dairy bacteriology of that time, H. Weigmann, E. Duclaux, and especially E. von Freudenreich at Berne. The latter, originally a lawyer, had not much knowledge of chemistry, so a very fruitful co-operation was soon established between him and Orla-Jensen.

Until 1901 Orla-Jensen worked with von Freudenreich, and during this period most of the problems of the bacteriology of Emmenthal cheese were solved. Not only did they succeed in finding the source of and in isolating the special lactic acid bacteria of this cheese but, primarily as a result of the chemical studies of Orla-Jensen, the propionic acid bacteria were traced and isolated for the first time, and their importance to the aroma and hole formation of the cheese was elucidated. The interest then centred upon the ripening processes of cheese. At that time it was a common belief, dating from the studies of Duclaux, that the breakdown of the proteins of cheese was caused by proteolytic spore-forming bacilli named *Tyrothrix*. But in Berne it was proved that the lactic acid bacteria were the main cause of this process, and it was later shown by Orla-Jensen that the proteolytic endo-enzymes liberated at the death of these bacteria were the active catalysts.

In the course of a few years Orla-Jensen had by these investigations placed himself in the forefront of dairy bacteriology, a position which he held until his death. Thus it was a matter of course that, after the death of von Freudenreich, he was appointed director of the Swiss experimental station of dairying at Liebefeld, near Berne. This post he held till 1906 and during that period a steady flow of papers appeared covering all problems within the dairy industry, e.g. butter defects, and the part played by rennet and bacterial enzymes in the ripening of cheese. In 1904 his doctoral thesis was published, on the types and origin of volatile fatty acids in cheese. This paper so much increased the interest in Orla-Jensen's work that in 1906 he was called to occupy a special chair at the Technical University of Denmark, and in 1908 he was appointed professor

of fermentation physiology and agricultural chemistry, later termed biotechnical chemistry. During the forty years he held this position, his work was of the greatest importance to the dairy and fermentation industries in Denmark and abroad.

Orla-Jensen now concentrated upon the classification of bacteria. Finding the old methods of classification, based as they were on purely morphological characters, quite inadequate and even misleading, he proposed in 1908 a rather revolutionary, 'natural' classification, based mainly upon physiological characters (nutritional demands, dissimilation products, etc.), flagellation and Gram-staining. He held that morphologically different bacteria may belong to the same systematic group, and this point of view has in later years gained more and more support in bacterial taxonomy. He was of the opinion that bacterial names should be descriptive, and often paid little attention to the priority of names, which gives us, as he said, a heavy burden of ill-chosen names and the difficulty of identifying any given strain with the very vague descriptions in the older literature.

At this time Orla-Jensen started his studies on the taxonomy of the lactic acid bacteria, which after ten years of very thorough investigation, resulted in his main publication, *The Lactic Acid Bacteria* (1919). In this work there were for the first time adequate descriptions of practically all known species of this important group including many new species. Furthermore, they were classified in an admirably comprehensive system which facilitated the identification of these bacteria. Following his earlier theories Orla-Jensen grouped both rod and sphere forms in the same family, because their physiological characters were exactly the same though the morphology of many species was variable. The long time taken to perform this work shows one of the factors that decide the permanent value of most of Orla-Jensen's publications. As a born scientist he combined an audacious and sure intuition with great perseverance and a highly critical sense which would not allow him to draw any conclusions that were not founded on exactly controlled experimental facts. Hence the many strains studied during these years were examined again and again to decide which characters are the most constant and therefore should be used as systematic criteria. It was found that the most stable characters were temperature relationships, the optical form of the lactic acid produced and the fermentation of a series of sugars and alcohols. In a supplementary volume of *The Lactic Acid Bacteria*, published in 1943, it was further shown that these characters had not changed in cultures which had been stored in the laboratory for thirty to forty years. Also of great importance was his demonstration that to identify the lactic acid bacteria it is necessary to supply them with an optimal source of nitrogen and cultivate them at optimal temperature.

This classical work has been the foundation of most later research in this field, research which has added little more of interest to our knowledge of the taxonomy of the lactic acid bacteria. Also it placed his laboratory as the centre of international research on this group of bacteria. It is true that in later years intensive investigations have added much to our knowledge of the exact nutritional requirements of the lactic acid bacteria, but in this field also Orla-Jensen was a pioneer contributing two important papers (1936, 1940) to the

elucidation of these questions. It must be said, however, that he was rather too early with these investigations, as many of the important growth substances were unknown at that time.

Orla-Jensen had a great interest in milk sanitation. As early as 1912, in collaboration with Chr. Barthel, he introduced the methylene-blue reduction test for the practical bacteriological grading of milk. As is well known this ingenious method has since been prescribed by law in many countries. Orla-Jensen also led a life-long fight for the pasteurization of milk. Most of his family died from milk infections when he was a child so this campaign was of the utmost personal importance to him; as a result most of the consumer milk in Denmark is now pasteurized.

Until the age of 76 years, Orla-Jensen was the leader of his laboratory, and even at this advanced age he had an abundance of ideas and carried them out with great energy. Thus, in these years, a comprehensive study was made of the intestinal flora in old people in order to re-examine the hypothesis of Metchnikoff as to the possible connexion between senility and intestinal flora. This work led to interesting conclusions showing that the secretion of hydrochloric acid in the stomach, which is always very low in senile people, is of prime importance, while the changes in the intestinal flora are a consequence of the changes in pH in the stomach. His last publication treated of the ensiling of cattle-fodder. Even here he had new ideas. He prepared an excellent silage from lucerne, rich in proteins, by adding large amounts of 24 hr. cultures of lactic acid bacteria in a mixture of whey and molasses so that the pH in the fodder decreased rapidly enough to check putrefaction.

In spite of failing strength Orla-Jensen worked with dairy problems to the last. As a result of his great experience in the processes of cheese-ripening he developed a method for the production of a rapidly ripening cheese with a specially sweet taste. The practical difficulties in the manufacture of this cheese were almost overcome when he died, but he did not have the satisfaction of seeing it established on a large scale.

The death of Orla-Jensen is a great loss to microbiology, and to all who knew him it is also a great personal loss. In combination with his great qualities as a scientist he had great common sense, and it was always enlightening to discuss problems with this man who was able to look at them from wider aspects and thus had the gift of knowing what was important and what was not. He was very industrious. In later years he was constantly writing or studying, and in his younger days he carried out most of the work in his laboratory himself, usually assisted by his wife, Mrs A. D. Orla-Jensen, C.E., who at work, as at home, played a very great part in his success.

Orla-Jensen received due recognition for his contributions; he was highly decorated with Danish and foreign orders, was Doctor of Science, *honoris causa*, honorary professor at several universities and member of the academies of science in many countries. He was elected an Honorary Member of this Society in 1949.

The death of Orla-Jensen is the end of a happy life, and to microbiology it means the finish of an epoch.

ERIK OLSEN

The Bacteriostatic Action of Phenol, Benzoic Acid and Related Compounds on *Bacterium aerogenes*

By R. G. H. BARBOUR and J. M. VINCENT
School of Agriculture, University of Sydney, New South Wales, Australia

SUMMARY: A method was developed that permitted the immediate and relatively uncomplicated study of the inhibition of *Bacterium aerogenes* during the logarithmic growth phase. With the ten aromatic compounds tested the linear growth rate was immediately established in the presence, as well as in the absence, of inhibitor.

The results with *Bact. aerogenes*, as well as some similar determinations with *Aspergillus niger*, using 50 % inhibition as criterion, suggest that in the phenols and in the acids, considered separately, there is a reasonably inverse relationship between inhibiting potency and water solubility. Substitutions that lead to an *ortho*-relationship between hydroxyl and carboxyl groups do not lessen but rather increase potency; *meta*- and *para*-substitutions of these groups lessen potency.

On the hypothesis that inhibition is proportional to the number of inhibitor molecules adsorbed at the surface of a susceptible metabolic system, potency can be analysed in terms of two factors: (*a*) the ability to adsorb on such a surface, which largely parallels 50 % inhibition; and (*b*) the degree of saturation of the surface with increasing inhibition, which is small for the phenols, large for the benzoic and the two dihydroxybenzoic acids, and intermediate for the monohydroxybenzoic acids.

Methods in use for the study of bacteriostatic action have been discussed in a recent communication from this laboratory (Murrell & Vincent, 1949), which also describes experience with full growth-curve methods, based substantially on those used by Hinshelwood and co-workers (Hinshelwood, 1944). Although the lengthening of the lag phase that can be demonstrated by these methods has its particular interest, inhibition during the logarithmic growth phase probably constitutes the simplest and most direct approach to the relationship between inhibitor and organism. If the full growth curve is used, the organisms must pass through a lag before reaching the logarithmic phase, so that they are in contact with the inhibitor for periods that vary with its concentration. A method has therefore been developed which exposes a large number of cells to different concentrations of the inhibitor without interruption of an established logarithmic growth phase. We record results obtained by this method with a series of simple aromatic compounds, together with data obtained by one of us (J. M. V.) for *Aspergillus niger* by the method already described (Vincent, 1947).

EXPERIMENTAL

Inhibitors. These comprised phenol, catechol, resorcinol, benzoic acid, *o*-, *m*- and *p*-hydroxybenzoic acids, methyl *p*-hydroxybenzoate, resorcylic (2,4-dihydroxybenzoic) acid and protocatechuic acid. All compounds were as used in an earlier investigation (Cavill, Phillips & Vincent, 1949) or were obtained from commercial sources and purified. Benzoic and the monohydroxybenzoic acids were used as sodium salts; the dihydroxybenzoic acids were neutralized with the calculated volume of NaOH immediately before use to avoid excessive colour development.

Organism. *Bacterium aerogenes* Type I, strain 32 (Murrell & Vincent, 1949) was used throughout.

Medium. Buffered glucose peptone solution of the following composition was used: Witte peptone (0·5 %, w/v), glucose (1 %), NaCl (0·5 %), K_2HPO_4/KH_2PO_4 buffer (0·2 M), ascorbic acid (0·1 %).

The initially clear medium essential for use in a photometer was obtained as follows:

(1) Peptone water. Witte's peptone (0·59 g.) and NaCl (0·59 g.) were dissolved with heating in 100 ml. water, filtered twice under pressure through cotton wool and high-grade filter paper, autoclaved and stored in screw-capped bottles.

(2) Phosphate buffer. 2 M-K_2HPO_4 and KH_2PO_4 were mixed in suitable proportions to give a pH of 7·0 and autoclaved.

(3) Glucose solution (20 %, w/v) was sterilized by Seitz filtration.

(4) Ascorbic acid (0·1 g.) was sterilized by exposure to 70 % ethanol which was subsequently removed by evaporation.

The components were mixed aseptically in the following proportions: peptone water, 85 ml.; phosphate buffer, 10 ml.; glucose solution, 5 ml.; ascorbic acid, 0·1 g. The mixture was stored in a cool room overnight and Seitz filtered immediately before use. All water used in the preparation of media, inhibitor dilutions and final rinsing of equipment was glass-distilled.

Inhibitor dilutions. In general, stock solutions were prepared by dissolving a known weight of inhibitor, sterilized by previous exposure to 70 % ethanol (except resorcylic acid which thus became very coloured), in a calculated volume of 0·2 M-phosphate (pH 7·0) to give a concentration ten times greater than the highest required in contact with the organisms. The range of concentrations was then provided for by adding to each of a series of aluminium-capped and optically matched tubes the requisite proportions of stock inhibitor solution and plain buffer, in each case to a total volume of 1 ml.; control tubes contained 1 ml. plain buffer. The subsequent addition of 9 ml. suspension of organisms provided the final concentrations stated in recording the results.

The low water solubility of methyl *p*-hydroxybenzoate made it necessary to dissolve this compound in 95 % ethanol. Measured samples of this solution were added to the tubes to give a range of concentrations, the ethanol was evaporated under reduced pressure and, to facilitate final solution, 0·1 ml. ethanol was added to each tube before the addition of 10 ml. bacterial suspension. Ethanol was, in these cases, added to the control tubes; in no case did it affect the organism's growth.

Inoculation, determination of growth rate and inhibition. The method of inoculation is designed to ensure that, at the time the bacterial suspension comes into contact with the inhibitor, the organisms are in, and remain in, the logarithmic phase. The method is exemplified as follows:

A series of subcultures comprises: loopful from slope to 10 ml. broth (at 5 p.m.), 1–10 ml. broth (9 a.m.), 1–10 ml. broth (11 a.m.), 10–140 ml. broth (12.30 p.m.). A sample of the last culture is withdrawn at a suitable time, the density measured photometrically and the additional time required to reach 85×10^6 cells/ml. estimated. At the calculated time, 9 ml. (or 10 ml. in the case of methyl *p*-hydroxybenzoate) of this suspension are added to each of the tubes. Throughout the procedure care is taken to ensure that the organisms are not exposed to any marked change in temperature; all media and glassware are warmed to 37°.

Growth can now be followed photometrically at 550 mμ., with the water bath (37·5° ± 0·5) set alongside the instrument, taking readings of each tube in turn. The tube to follow that being read is shaken to ensure even distribution, and in time to allow bubbles to disperse from the liquid. Units of cell mass are determined from optical density by means of a standard curve for the organism.

Where a linear logarithmic increase with time is obtained, the growth rate can be calculated in the usual way and inhibition expressed as percentage inhibition $= \frac{k_c - k_i}{k_c} \times 100$, where k_c = control growth rate and k_i = growth rate in presence of inhibitor.

Inhibitors that colour the medium can, within reason, be studied by this method provided that the colour correction is not affected by the organism's growth. The addition of ascorbic acid prevented progressive colour development which would otherwise occur in the cases of catechol and protocatechuic acid due to quinone formation at pH 7. Ascorbic acid was added in all tests in the interests of uniformity of the medium.

At the time of adding the bacterial suspension to the inhibitors, a tube of the suspension was set aside in the cool room and its pH measured the following morning. In all cases the value was pH 6·9 and, since the medium was strongly buffered and there were no trends in growth rates for acid inhibitors (as would be expected for appreciable change in pH), this value has been taken as representative of the medium throughout the experiments.

Statement of results. Besides comparing substances in terms of the reciprocal of the millimolar concentration giving 50 % inhibition of growth rate (I_{50}), dosage-response data are interpreted in terms of a hypothetical adsorption relationship between organism and inhibitor (Phillips & Vincent, 1948).

RESULTS

Nature of growth curves. Representative curves with and without inhibitor are shown in Fig. 1. These are typical of those found throughout the present work; there is no sign of any lag or departure from linearity within the 1–2 hr. used for the establishment of the curves.

Dosage-response curves. The curves obtained by plotting inhibition directly against concentration can be interpreted for most of the inhibitors as passing through the origin (curves *A* and *C*, Fig. 2, being typical). However, resorcinol (curve *B*) and methyl *p*-hydroxybenzoate (curve *D*) seem to cut the abscissa at the right of the origin, indicating either that the organism can neutralize the action of a small amount of the inhibitor or that a threshold concentration must be reached before antibacterial action occurs (cf. Hinshelwood, 1946, fig. 25; and Vincent's (1947) experience with *Penicillium roqueforti* and *Byssochlamys fulva*).

Dosage-response data for the substances not included in Fig. 2 are given in Table 1. Each inhibition value represents the result in a single tube, all concentrations of an inhibitor being tested at the one time. Experience in an exploratory series of tests generally agreed with the later, more accurate, findings.

Comparison of inhibiting potency

The series of compounds tested in the present work, and against *Aspergillus niger*, are compared in cols. 1 and 2 of Table 2 on the basis of I_{50} expressed more conveniently in its logarithmic form. The values for *A. niger* differ in some detail

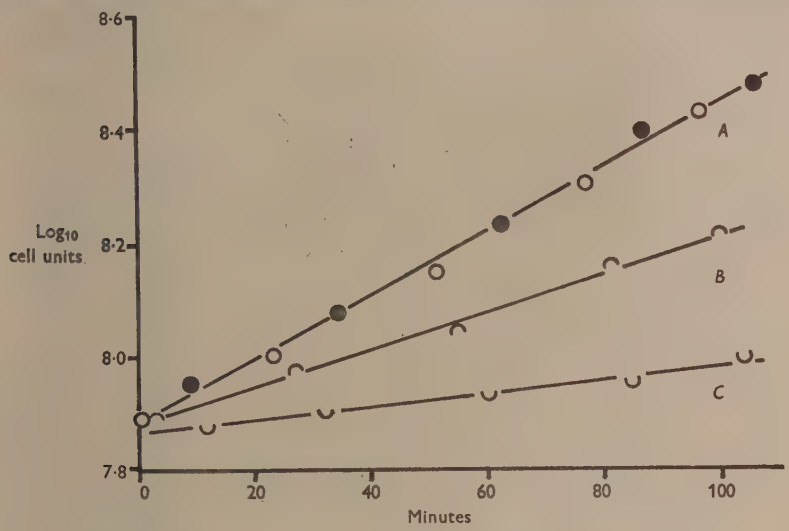


Fig. 1. Representative growth curves. *A*, **control, two experiments;** *B*, **phenol, 6 mM.;** *C*, **phenol, 12 mM.**

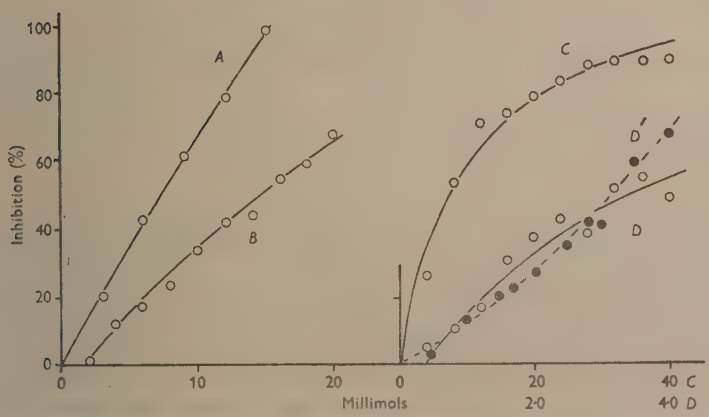


Fig. 2. Dosage-response curves. *A*, phenol; *B*, resorcinol; *C*, benzoic acid; *D*, methyl *p*-hydroxybenzoate; *D′*, methyl *p*-hydroxybenzoate by full growth method (Murrell & Vincent, 1949).

from those quoted by Cavill *et al.* (1949) and represent more exact values. They are, however, of the same order, and the earlier conclusions are substantially valid.

Before comparing substances and taking account of the effect of substitution it is necessary, particularly with *Bact. aerogenes* tested at pH 6·9, to consider the likely effect of anionic dissociation, which is generally found to lessen inhibiting potency. At pH 6·9 the carboxylic acids will be almost entirely dissociated, whereas in tests against *Aspergillus niger* at pH 3·5 practically all

Table 1. *Dosage-response data for resorcinol, catechol, monohydroxybenzoic acids and resorcylic acid against* Bacterium aerogenes

y = percentage inhibition observed: Y = percentage inhibition predicted from the adsorption relationship of equation (3).

Concentration (mM.)	Inhibition (%)					
	Resorcinol		Catechol		Salicylic acid	
	y	Y	y	Y	y	Y
2	—	—	—	—	32·8	32·8
4	11·6	10·4	18·3	18·4	56·7	53·3
6	17·4	18·3	26·0	26·3	63·9	67·4
8	23·8	25·9	34·0	33·6	76·0	77·5
10	33·8	33·2	41·1	40·4	86·0	85·3
12	41·5	40·1	51·1	46·6	92·5	91·4
14	43·9	46·9	47·8	52·3	—	—
16	54·1	53·3	54·0	57·7	—	—
18	59·2	59·5	65·5	62·6	—	—
20	67·9	65·5	68·5	67·3	—	—
	m-Hydroxybenzoic acid		*p*-Hydroxybenzoic acid		Resorcylic acid	
	y	Y	y	Y	y	Y
10	17·3	15·2	21·3	20·9	39·1	39·5
20	21·9	28·4	35·9	38·5	50·0	53·7
30	42·2	39·9	53·9	53·7	69·8	61·1
40	48·9	50·1	68·2	66·9	70·8	65·6
50	61·4	59·2	86·0	78·3	59·7	68·6
60	78·0	67·3	85·0	88·5	70·1	70·8
70	79·7	74·6	95·4	97·5	75·7	72·5
80	81·5	81·3	—	—	—	—
90	85·7	87·3	—	—	—	—
100	86·0	92·8	—	—	—	—

the inhibitor will be in the non-ionized state. It is perhaps surprising, therefore, that the difference in log I_{50} between the two sets of experiments is so slight. It certainly appears unjustifiable to apply a correction based on the assumption that all activity resides in non-ionized molecules of the bulk phase. The extent of such corrections is shown in Table 2 (cols. 7 and 8), and if applied in the case of *Bact. aerogenes* to *p*-hydroxybenzoic acid would give log I_{50} of 1·0 compared with 0·2 for the methyl ester. This would quite reverse our experience with *Aspergillus niger* and general knowledge of the effect of esterification. By the same correction log I_{50} for salicylic acid would attain the unlikely value of 3·4. To avoid such ridiculous estimates it would be necessary to regard the ionized form at least a third to a half as active as the non-ionized or to picture the inhibitor acting at a biological surface for which the pH is much lower than that of the bulk phase. Comparisons are made, therefore, without adjustment.

Log I_{50} data are related to water solubility (expressed as $-\log S_w$, where S_w is molar water solubility) in Fig. 3. It will be seen that among the phenols and acids separately (treating the hydroxyacids as substituted acids rather than substituted phenols; cf. Cavill *et al.* (1949)) there is a fair inverse agreement between water solubility and inhibitory effect. As between phenols and acids, the agreement breaks down.

Table 2. *Inhibition data for* Bacterium aerogenes *and* Aspergillus niger

BA = *Bact. aerogenes*; *AN* = *A. niger*.
log I_{50} = −log millimolar concentration for 50 % inhibition.
A/K = ratio of effective area (A) and relative inhibition (K) per molcule adsorbed.
log B = log of 'biological adsorbability' of the inhibitor.
log S_w = log water solubility (Cavill *et al.* 1949).

	log I_{50}		A/K		log B		−log proportion non-ionized		−log S_w
Inhibitor pH...	*BA* 6·9	*AN* 3·5	*BA* 6·9	*AN* 3·5	*BA* 6·9	*AN* 3·5	*BA* 6·9	*AN* 3·5	
Phenol	−0·87	−0·52	0·0005	0·0051	−2·47	−0·99	—	—	0·2
Resorcinol	−1·18	−1·75	0·0026	0·0054	−1·96	−2·18	—	—	−0·8
Catechol	−1·11	−1·17	0·0050	0·0111	−1·60	−1·08	—	—	−0·6
Salicylic acid	−0·56	−0·39	0·0070	0·0109	−0·82	−0·32	4·0	0·6	1·8
m-Hydroxybenzoic acid	−1·60	−1·41	0·0047	0·0109	−2·10	−1·33	2·8	0·2	1·2
p-Hydroxybenzoic acid	−1·44	−1·65	0·0040	0·0120	−2·05	−1·48	2·4	0·1	1·4
Methyl *p*-hydroxybenzoate	−0·53	−0·18	0·0090	—	−0·57	—	0·7	—	—
Benzoic acid	−0·88	−0·30	0·0086	0·0138	−1·00	0·04	2·7	0·1	1·6
Resorcylic acid	−1·22	−1·10	0·0119	0·0150	−1·02	−0·63	3·7	0·4	1·8
Protocatechuic acid	*	−2·0	—	0·0146	—	−1·58	—	—	0·9

* Preliminary tests for protocatechuic acid showed that against *Bact. aerogenes* this acid exhibited a very low order of activity. Shortage of material prevented further testing.

For comparative purposes it is convenient to introduce the value (Table 3)

$$\Delta \log I_{50} = \log I_{50} \text{ of derived substance } - \log I_{50} \text{ of parent substance.}$$

When this is done it is found that hydroxyl and carboxyl substitutions, other than those providing an *ortho*-relationship between hydroxyl and carboxyl, lead to a consistent loss of inhibitory effectiveness (groups *b* to *d*). The *ortho*-relationship results most commonly in a gain of activity or in little loss (group *a*).

Further interpretation of dosage-response relationships

In an earlier paper (Phillips & Vincent, 1948) fungistatic inhibition was interpreted as being related to adsorption expressed as the Langmuir isotherm in which

$$A\theta = \frac{Bx}{1+Bx}, \qquad (1)$$

where θ = number of molecules adsorbed/unit area, A is the effective area occupied at the biological surface by each molecule adsorbed, x is the millimolar concentration of substance being adsorbed, and B is the ratio of constants of adsorption and desorption.

If inhibition (y) is regarded as proportional to the number of molecules adsorbed,

$$y = K\theta, \tag{2}$$

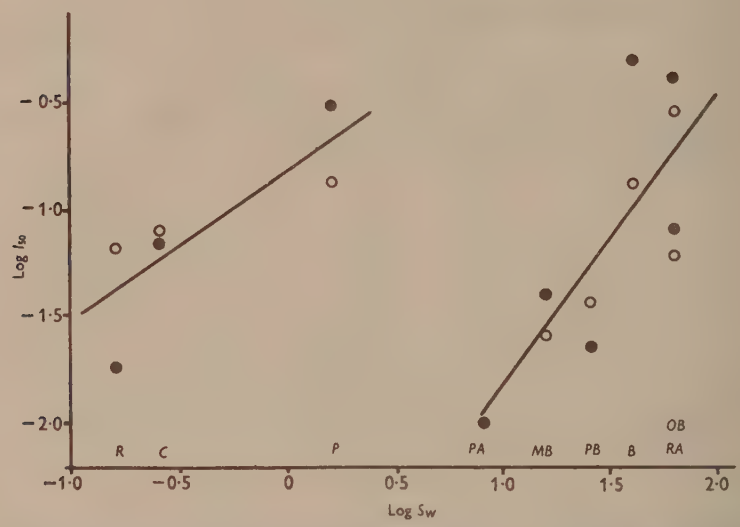


Fig. 3. Relationship between inhibitory action ($\log I_{50}$) and water solubility ($-\log S_w$). *P*, phenol; *R*, resorcinol; *C*, catechol; *B*, benzoic acid; *PB*, *MB* and *OB*, *p*-, *m*- and *o*-hydroxybenzoic acids; *RA*, resorcylic acid; *PA*, protocatechuic acid. Open circle, *Bact. aerogenes*; filled circle, *Aspergillus niger*.

where K expresses the relative inhibitory effectiveness per molecule adsorbed. From (1) and (2),

$$y = \frac{K}{A}\frac{Bx}{1+Bx}. \tag{3}$$

The validity of the assumption is supported by the agreement between experimental points and the values predicted on the basis of this equation (curves *A* and *C*, Fig. 2 and Table 1). The remaining two inhibitors can also be interpreted in terms of the same relationship if, as already noted, a reasonable adjustment is made for 'tolerance'. Apart from the general agreement between the inhibition-concentration curve and the form of the adsorption isotherm, it is possible by the use of the present bulk inoculum technique to see that a steady state condition is quickly established between organism and inhibitor (indicated by the linear curves in Fig. 1) which is in accordance with the postulate that growth-rate inhibition is being controlled by an adsorption step likely to reach a fairly rapid equilibrium.

A linear form of equation (3) expressed as

$$\frac{x}{y}=\frac{A}{KB}+\frac{A}{K}x \tag{4}$$

has certain practical and theoretical advantages in the statistical fitting of experimental data and the evaluation of constants. In testing this linear form,

Table 3. *Influence of substitutions of hydroxyl and carboxyl groups on criteria of inhibition. Comparison of parent and derived compounds*

	Compounds		Log ratio				
			Inhibiting power ($\Delta \log I_{50}$)		Biological adsorbability ($\Delta \log B$)		Water solubility ($-\Delta \log S_w$)
	Parent	Derived	*BA*	*AN*	*BA*	*AN*	
(*a*) *Ortho*- −COOH and −OH	Phenol	Salicylic acid	+0·31	+0·13	+1·65	+0·67	+1·6
	Benzoic acid	Salicylic acid	+0·32	−0·09	+0·18	−0·36	+0·2
	Resorcinol	Resorcylic acid	−0·04	+0·65	+0·94	+1·55	+2·6
	p-Hydroxy-benzoic acid	Resorcylic acid	+0·22	+0·55	+1·03	+0·85	+0·4
(*b*) *Ortho*- −OH and −OH	Phenol	Catechol	−0·24	−0·65	+0·87	−0·09	−0·8
	p-Hydroxy-benzoic acid	Protocatechuic acid	—	−0·35	—	−0·10	−0·5
	m-Hydroxy-benzoic acid	Protocatechuic acid	—	−0·59	—	−0·25	−0·3
(*c*) *Meta*- −COOH and −OH	Phenol	Resorcinol	−0·31	−1·23	+0·51	−1·19	−1·0
	Phenol	*m*-Hydroxy-benzoic acid	−0·73	−0·89	+0·37	−0·34	+1·0
−OH and −OH	Benzoic acid	*m*-Hydroxy-benzoic acid	−0·72	−1·11	−1·10	−1·37	−0·4
	Catechol	Protocatechuic acid	—	−0·83	—	−0·50	+1·5
(*d*) *Para*- −COOH and −OH	Phenol	*p*-Hydroxy-benzoic acid	−0·57	−1·13	+0·42	−0·49	+1·2
	Benzoic acid	*p*-Hydroxy-benzoic acid	−0·56	−1·35	−1·05	−1·52	−0·2
−OH and −OH	Salicylic acid	Resorcylic acid	−0·66	−0·71	−0·20	−0·31	0

BA = *Bact. aerogenes*; *AN* = *Aspergillus niger*. Δ = log value derived − log value parent substance.

substances whose curve passes through the origin in Fig. 2, give good agreement with straight lines of positive slope (curves *A* and *C* of Fig. 4). However, those showing tolerance, as would be expected from the nature of the equation for the linear relationship, give x/y values for the lower values of x that appear to be very high and may give an apparent negative slope (resorcinol, unadjusted values, Fig. 4) that has no meaning in terms of an adsorption isotherm. When a tolerance value (estimated from a freehand drawing of the arithmetic curve) is deducted from x to give a series of x' values, x'/y gives a reasonably linear relationship with positive slope (curves *B* and *D*, Fig. 4). In these two cases adjusted values have been used for the calculation of adsorption constants.

A/K, expressing the relationship between the area occupied and percentage inhibition/molecule adsorbed, is calculated as the slope of the line of best fit in equation (4). Data for *Bact. aerogenes* and *Aspergillus niger* are given in cols. 3 and 4 of Table 2. Any ionization correction, in so far as it changes the numerical value of x on both sides of equation (4), will be without effect on the calculation

of A/K. Although the values for *A. niger* appear generally higher than for *Bact. aerogenes* they agree in showing certain trends related to chemical structure. Using the two organisms together as a basis for the testing of such trends, it is possible to show that phenol and resorcinol have low values that contrast with benzoic acid and the dihydroxybenzoic acids at the other end of the scale. Catechol and the monohydroxybenzoic acids occupy intermediate positions.

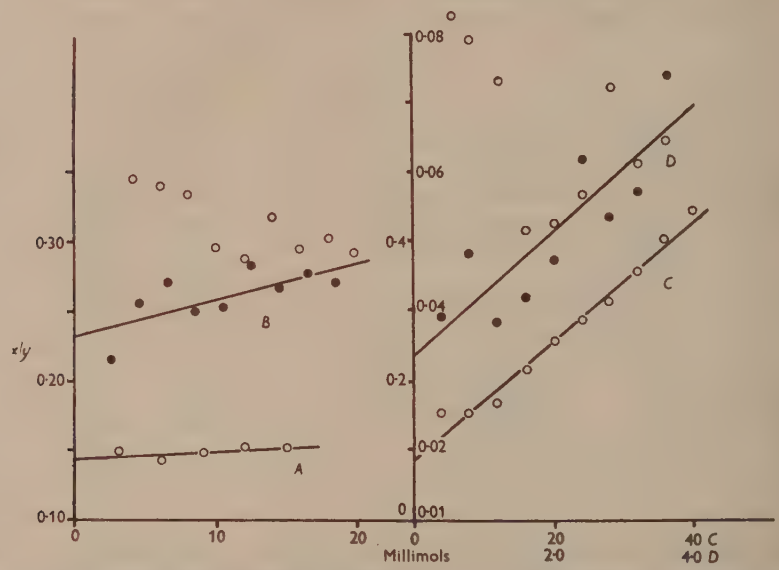


Fig. 4. Dosage-response curves—linear form. *A*; phenol; *B*, resorcinol; *C*, benzoic acid; *D*, methyl *p*-hydroxybenzoate. Ordinate: concentration (x)/inhibition (y). Lines *B* and *D* fitted to adjusted values (full circles).

The reciprocal of A/K gives a direct measure of the relative effectiveness per molecule adsorbed on the basis of the space it occupies. Its influence is seen in Fig. 5 in which the effect of the biological adsorbability constant (B) has been removed from the collection of curves by plotting X in place of x so that for each substance, $X = Bx$. The low A/K value for phenol results in a practically straight line within the range of observable inhibition, so that attainment of a high degree of inhibition occurs with very little occupation of the surface. The other parent substance (benzoic acid) has a high value for A/K so that the curve flattens over at about 50 % inhibition, and very much higher concentrations are required to provide 100 % inhibition. From equation (3), since $\frac{Bx}{1+Bx} \to 1$ as the surface approaches saturation, K/A sets the upper limit of y, so that A/K must $\leqq 0{\cdot}01$, if 100 % inhibition be theoretically attainable. From the present data it will be seen that resorcylic acid cannot be expected to provide 100 % inhibition of *Bact. aerogenes*, 84 % being the theoretical

upper limit. The intermediate behaviour of the remaining substances is also well shown in Fig. 5.

Solution of A/K permits determination of B from equation (4) and data for this criterion, expressed as log B, are included in Table 2. For reasons similar to those discussed in connexion with the I_{50} criterion, no correction has been

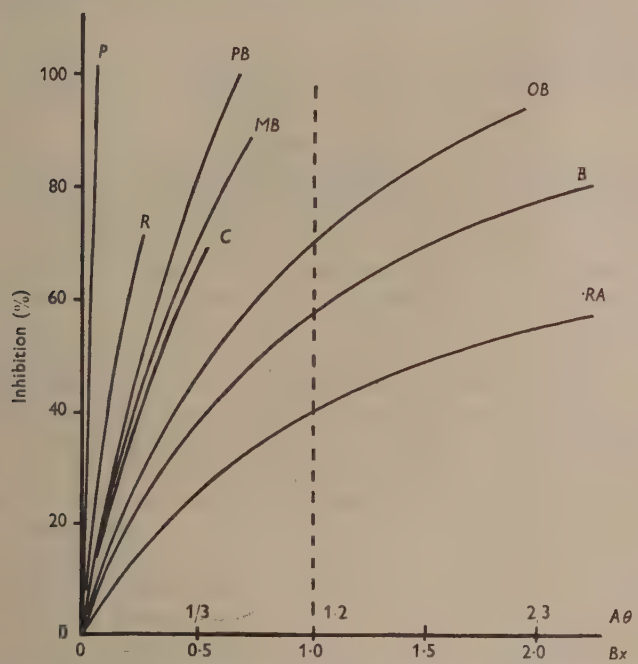


Fig. 5. Influence of A/K on inhibition relative to space occupied. *P*, phenol; *R*, resorcinol; *C*, catechol; *B*, benzoic acid; *PB*, *MB* and *OB*, *p*-, *m*- and *o*-hydroxybenzoic acids; *RA*, resorcylic acid; $A\theta$ = proportion of 'biological space' occupied; Bx = 'biological adsorbability' × millimolar concentration.

applied for ionization. Amongst the phenols there is fair agreement between the two organisms except for phenol itself. In this case the value against *Bact. aerogenes* might be rather low, and it is a disadvantage of this constant that errors in A/K are likely to cause errors in A/KB that will accentuate the variability of log B. This is particularly liable to happen for lower values of A/K. Having regard to the differences in pH, the trends within the group of acids agree quite well. Apart from the low value for phenol against *Bact. aerogenes*, there is, as for log I_{50}, a fair measure of agreement between B and solubility figures among the phenols and acids separately, but no agreement between the groups.

Δ log B can now be used as for Δ log I, the results being tabulated in Table 3. Rather more consideration is required than for Δ log I particularly in that comparisons with phenol are likely to be discrepant between the two

organisms because of the very low value for phenol against *Bact. aerogenes*. In group (*a*) leading to *ortho*-hydroxyl and carboxyl, the relationship between the two criteria is quite close for benzoic acid → salicylic acid but the other three cases involve a proportionately greater increase in biological adsorbability than in the log I_{50} criterion. The difference has its explanation in the increased A/K characteristic of the monohydroxybenzoic acids compared with phenol, and resorcylic acid compared with *p*-hydroxybenzoic acid and resorcinol. In the remaining groups there is, excluding comparisons involving phenol with *Bact. aerogenes*, fair agreement between the trends of log B and log I.

DISCUSSION

Although the mass inoculum technique developed in the present work is rather exacting, has the disadvantage of limiting the number of tubes that can be handled at one time and, like any spectrophotometric method, is affected by colour in the inhibitor, nevertheless it seems well suited for the observation of bacteriostasis within a very short time of initial exposure and without interruption of the logarithmic growth. It is likely to be subject to a minimum of uncontrolled side effects as is indicated by the comparison in Fig. 2 of data for methyl *p*-hydroxybenzoate obtained by the small inoculum, full growth-curve method (Murrell & Vincent, 1949) with the curve obtained by the new method. It seems reasonable to conclude that the departure of the curve based on the earlier method from the typical adsorption form reflects the more complex nature of the relationship being studied, including some loss of viability at higher concentrations and an appearance of enhanced bacteriostasis.

The present paper also illustrates two approaches to the use of dosage-response data. In the first instance we have compared substances at a convenient inhibitory level and further we have interpreted the results on the basis of an adsorption relationship. The second approach permits an analysis of the biological effect in terms of biological adsorbability, and the effectiveness relative to the area occupied by each molecule adsorbed (A/K). Generally there is fair agreement between the inhibitory index (I_{50}) and biological adsorbability (B); but the former can be regarded as a resultant of A/K operating on B and some disagreement is to be expected. The phenols are characterized by a smaller A/K than benzoic acid, the monohydroxybenzoic acids are intermediate, but the dihydroxybenzoic acids have an A/K as great as the parent acid. Such differences in A/K might be due to intrinsic differences in the effectiveness per molecule adsorbed (K) or to a change in the effective area of the biological surface occupied by the molecule (A) or to changes in both factors.

The authors are indebted to Mr C. Warner for preparing some of the inhibitors and to Mr J. Phillips for critical discussion of the text. The work was assisted by a grant from the Commonwealth Research Fund.

REFERENCES

CAVILL, G. W. K., PHILLIPS, J. N. & VINCENT, J. M. (1949). Relationship between fungistatic activity and structure in a series of simple aromatic compounds. *J. Soc. chem. Ind., Lond.*, **68**, 12.

HINSHELWOOD, C. N. (1944). Bacterial growth. *Biol. Rev.* **19**, 150.

HINSHELWOOD, C. N. (1946). *The Chemical Kinetics of the Bacterial Cell.* Oxford: Clarendon Press.

MURRELL, W. G. & VINCENT, J. M. (1949). The esters of 4-hydroxybenzoic acid and related compounds. IV. The bacteriostatic action of *n*-alkyl-4-hydroxybenzoates. *J. Soc. chem. Ind., Lond.* (Accepted for publication.)

PHILLIPS, J. N. & VINCENT, J. M. (1948). Dosage/response relationships in mould inhibition. *Nature, Lond.*, **161**, 210.

VINCENT, J. M. (1947). The esters of 4-hydroxybenzoic acid and related compounds. I. Methods for the study of their fungistatic properties. *J. Soc. chem. Ind., Lond.*, **66**, 149.

(*Received* 8 *May* 1949)

The Antibiotic Properties of Fifty-two Strains of *Fusarium*

By MARGARET S. LACEY
Imperial College of Science and Technology, London

SUMMARY: Culture fluids of fifty-two strains of *Fusarium* spp. were examined for antibacterial activity. Four groups were distinguished: (*a*) six strains equally active against a strain of *Staphylococcus aureus* and *Mycobacterium phlei* strain 1; (*b*) sixteen strains much less active against *Staph. aureus* than against *Myco. phlei* 1; (*c*) fourteen strains inactive against *Staph. aureus*, active against *Myco. phlei* 1; (*d*) sixteen strains inactive against both *Staph. aureus* and *Myco. phlei* 1, of which six were active against *Myco. phlei* strain 2.

Examination of pure antibiotic substances isolated from the culture fluids of nine of the strains showed them to be both bacteriostatic and bactericidal; they were very active against mycobacteria and effective to a lesser degree against other Gram-positive bacteria. They were ineffective against Gram-negative bacteria and fungi.

Earlier papers described the production of an antibiotic pigment, javanicin, from *Fusarium javanicum* (Arnstein, Cook & Lacey, 1946*a*, *b*), and the isolation of five antibiotic substances from strains of *F. lateritium*, *F. fructigenum*, *F. sambucinum* and *F. avenaceum* respectively (Cook, Cox, Farmer & Lacey, 1947). The last paper also recorded the preliminary sorting of twenty-two *Fusarium* strains into four groups according to the type of antibiotic activity of the culture fluids. Thirty more strains have now been examined, making fifty-two in all, and in some cases attempts have been made to isolate the active principles.

EXPERIMENTAL

Methods of testing for antibiotic activity

For general routine examinations the *Fusarium* cultures were grown at 25° in 150 ml. conical flasks containing 50 ml. of a medium consisting of Bacto-tryptone or Eupeptone, 10 g.; sodium chloride, 5 g.; glucose, 40 g.; in 1 l. of water. This medium was the most satisfactory for the preliminary examination, because *Fusarium* strains capable of antibacterial activity always produced active material in this medium, though not always of the highest possible potency. At frequent intervals from the 3rd to the 28th day samples were withdrawn by sterile pipettes from each flask and tested for antibacterial activity.

Two bacterial strains, *Staphylococcus aureus* and *Mycobacterium phlei* strain 1, were used as routine test organisms. In many cases *Myco. phlei* strain 2, *Streptococcus pyogenes*, *Bacillus subtilis* and *Corynebacterium fascians*, representing different types of Gram-positive bacteria, and the Gram-negative species *Escherichia coli*, *Pseudomonas fluorescens* and *Ps. aeruginosa* were also used. Activity against the Gram-positive species varied with different *Fusarium* strains, but all the strains were inactive against the Gram-negative bacteria.

The *Fusarium* strains were usually much more effective against *Myco. phlei* than against *Staph. aureus*, *Strep. pyogenes* or *B. subtilis*. The two strains of *Myco. phlei* varied considerably in their susceptibility to the various *Fusarium* strains; *Myco. phlei* 2, which in tests against pure antibiotic substances resembled *Myco. tuberculosis* more closely than did *Myco. phlei* 1, was sometimes more and sometimes less susceptible than *Myco. phlei* 1. The *Fusarium* culture fluids were strongly active against *C. fascians* (a plant parasite of diphtheroid type).

Two methods of testing for antibacterial activity were used: (*a*) the agar-plate method, in which the culture fluids were filled into holes cut with a cork-borer in nutrient agar plates seeded with the test bacterium; (*b*) by serial dilution in broth. Plates and tubes were incubated at 37° (30° for *C. fascians*), and the results read the next day when *Staph. aureus*, *Strep. pyogenes* or *B. subtilis* was the test organism, and after 2 or 3 days with *Myco. phlei* and *C. fascians*. Most of the tests were made by the broth-dilution method but the agar-plate method was useful for determining the first appearance of antibiotic activity.

Measurement of potency

Antibiotic potency is expressed as the highest dilution of fluid tested which completely inhibited the growth of the test bacteria; thus a potency of 1/200 means that 1/200 was the maximum inhibiting dilution of a given culture fluid.

Development of antibiotic activity

In general, culture fluids were feebly active on the fourth day and reached their maximum potency at 10–14 days. With some strains (e.g. *F. lateritium*, *F. sambucinum*) the maximum potency was maintained with little change for a month or more. For others, notably in members of group C (see below), the maximum potency was maintained for 24–48 hr. only, followed by a rapid fall to zero.

Except with *F. javanicum* cultures, where potencies of 1/100 to 1/200 were obtained, the potency of the crude culture fluids was low, 1/20 to 1/25 against *Myco. phlei* usually being the maximum. Those antibiotics (lateritiin, etc.) which were isolated were very sparingly water-soluble; this may account for the small quantity present in the culture fluids.

All the *Fusarium* cultures produced acid at first, followed later by alkali. Thus an initial pH value of 6·5 would fall to pH 4–5 during the first few days, after which there would be a gradual rise to pH 8–8·5. There was no correlation between pH value and antibiotic production.

The fifty-two *Fusarium* strains were sorted into four main groups according to the activity of the crude culture fluids against *Staph. aureus* and *Myco. phlei* 1:

Group A: Six strains equally active against *Myco. phlei* 1 and *Staph. aureus*.

Group B: Sixteen strains considerably more active against *Myco. phlei* 1 than against *Staph. aureus*.

Group C: Fourteen strains active against *Myco. phlei* 1, inactive against *Staph. aureus.*

Group D: Sixteen strains inactive against both *Myco. phlei* 1 and *Staph. aureus.*

Table 1 summarizes the results of testing the crude culture fluids of the fifty-two *Fusarium* strains. In no case was the test positive against *Staph. aureus* and negative against *Myco. phlei* nor was there ever greater activity against *Staph. aureus* than against *Myco. phlei* 1.

Table 1. *Activity of fifty-two* Fusarium *strains against* Mycobacterium phlei 1 *and* Staphylococcus aureus

Fusarium strains	Maximum inhibitory dilution against *Myco. phlei* 1	Maximum inhibitory dilution against *Staph. aureus*
Group A		
F. javanicum	1/200	1/200
F. dianthi 1 and 2	1/40	1/40
F. 6	1/10	1/10
F. 157	1/40	1/40
F. 64	1/50	1/50
Group B		
F. lateritium, four strains	1/20	1/5
F. sambucinum 1	1/25	1/5
F. avenaceum	1/20	<1/5
F. fructigenum, *F.* 135, *F.* 136, *F.* 137, *F.* 138	1/20	Trace or none, very transitory
F. dianthi 3, *F. lini*, *F. culmorum* 1, *F. moniliforme-subglutinans* 1, *F.* 63	1/5	Trace
Group C		
F. 91, *F.* 128, *F.* 131	1/40	0
F. 72, *F.* 117, *F.* 126, *F.* 130, *F.* 133	1/20	0
F. culmorum 2, *F. poae*, *F.* 101, *F.* 125	1/10	0
F. sambucinum 2, *F.* 129	1/5	0
Group D		
F. aquaeductum, *F. culmorum* 3 *F. oxysporum cubense* *F. moniliforme-subglutinans* 2 *F. dimerium*, *F. caeruleum* *F. sambucinum* 3, *F.* 10, *F.* 88, *F.* 94, *F* 95, *F.* 96, *F.* 97, *F.* 124, *F.* 127, *F.* 132	0	0

F. numbers refer to unidentified *Fusarium* strains.

Group A. Although the six strains were alike in producing equal activity against *Myco. phlei* 1 and *Staph. aureus*, the antibiotic substances produced by the different strains appeared to differ. *F. javanicum* is the only member of this group from which a pure antibiotic substance was isolated, but enough progress was made in the extraction of the active principles from the other strains to demonstrate their chemical, as well as their biological, diversity. *F. javanicum* differed from the other fifty-one strains in producing a soluble blood-red antibacterial pigment, javanicin (Arnstein *et al.* 1946*a*,*b*). Many of the other strains developed red, orange or yellow pigmentation of the mycelial

felt and sometimes a yellow pigment diffused into the medium, but this was inactive and there was no correlation between pigment production and antibiotic activity.

F. dianthi, strains 1 and 2, and *F.* 157 (?*F. pisi*, isolated from a foot-rot of peas) were similar in antibiotic activity. They were equally active against *Staph. aureus* and the two strains of *Myco. phlei*, and less active against *Strep. pyogenes* and *B. subtilis*. After the maximum potency was reached on the 7th to the 11th day there was a somewhat rapid fall to 1/5, which was still shown by month-old cultures. T. H. Farmer, who attempted the extraction of the antibiotics from these cultures, was unable to isolate a pure substance, but his results suggested the presence of two active principles, one of which might be a peptide.

Strain *F.* 64, from potato-rot, is of interest on account of the erratic and peculiar character of the antibiotic production. Repeated tests, both of the parent culture and of single spore isolations from the parent, exhibited the same phenomena. When *F.* 64 was grown on the standard Eupeptone medium, a potency of 1/10 on the 4th day was followed by a fall to zero on the 6th or 7th day. A second rise in activity and occasionally a second less marked fall then occurred, the maximum potency (occasionally up to 1/50) being reached about the 11th day. A slow decline followed, usually with slight further rises and falls, until by about the 21st day the culture fluids were inactive. In Czapek-Dox medium there was no activity until the 9th day; a maximum potency of 1/10 was reached by the 11th day followed by an irregular rise and fall in strength from day to day similar to the results with the Eupeptone medium. The addition of 0·2 % Yeastrel to the Czapek-Dox medium almost suppressed antibiotic production. Yet another type of activity developed in a 0·5 % Marmite 1 % glucose medium. No activity against *Staph. aureus* was developed, but a potency of 1/25 against *Myco. phlei* was reached on the 10th day: no day to day irregularities occurred in this medium.

A second peculiar phenomenon was repeatedly observed in agar-plate tests of *F.* 64 culture fluids against *Staph. aureus*. Instead of the usual clear and sharply defined zone of inhibition surrounding the cup there were frequently two zones of inhibition, an inner zone and an outer one, somewhat narrower, separated by a ring of very heavy staphylococcal growth: sometimes there was only partial inhibition of growth in the outer zone. This suggests interference with the inhibition mechanism at a certain critical concentration of the antibiotic.

Group B. The sixteen strains in this group were very similar in antibiotic activity against *Myco. phlei* 1. The activity appeared on the 5th or 6th day, reached a maximum at 12–14 days and was maintained at this potency for at least 1 month. The strains were tested on a variety of media to find the optimum conditions for antibiotic production. A medium containing Bactotryptone always gave the best results, but as the supply of this material was insufficient for the large-scale cultivation necessary for chemical work, substitutes were tested; of these Eupeptone was found to be the most satisfactory. The strains varied in their optimum glucose requirements and *F. sambucinum* was alone in producing a potency of 1/20 in a Bactotryptone medium without glucose.

Culture fluids active against *Myco. phlei* 1 at 1/20 dilution were usually effective against *Staph. aureus* at 1/5, but *F. fructigenum* and four strains isolated from cob-nuts (*F.* 135, *F.* 136, *F.* 137 and *F.* 138) were very feebly active against *Staph. aureus* from the 4th to the 7th day only, and by the time the maximum potency (1/20) against *Myco. phlei* was reached the culture fluids were completely inactive against *Staph. aureus*. Later it was found that impure preparations of the antibiotics from the culture fluids of these five strains were also inactive against *Staph. aureus*, although they increased in potency against *Myco. phlei* as purification proceeded. However, when the pure antibiotics were finally obtained, they were as potent against *Staph. aureus* as sambucinin and more potent than avenacein.

A second unexplained phenomenon was the erratic inhibition of *Myco. phlei* 1 in dilution tests of crude extracts of *F. fructigenum*. For example, in one test there was thick growth of *Myco. phlei* in the 1/50 dilution but complete inhibition in the 1/100 and 1/200 dilutions. This phenomenon occurred too frequently to be attributable to errors in testing. It occurred only with the impure preparation of the antibiotic; the pure substance behaved consistently. In these cases, as in *F.* 64 of group A, some substance appears to be present in the culture fluids which persists as an impurity in the extracts and which interferes with the inhibiting mechanism at certain concentrations of the antibiotic. Pure crystalline antibiotic substances were isolated from eight strains in this group (see below).

Group C. The antibiotic activity and chemical behaviour of these fourteen strains were so markedly different from those of group B as to justify their assignment to a separate group. The culture fluids in any medium and after any period of growth were completely inactive against *Staph. aureus* and the active principles were markedly unstable. Maximum potency, and the time taken to reach it, varied with different strains, but in every case there was a rapid rise and fall in activity against *Myco. phlei* 1, the maximum potency often being maintained for 24–48 hr. only; after 14–21 days the fluids were almost or entirely inactive. Attempts to isolate the active principles by my colleagues T. H. Farmer and S. F. Cox showed that the antibacterial substances produced by these strains were chemically different from the lateritiin group of antibiotics. Also, although these substances have not been obtained in a pure state, the marked differences in the chemical, as well as the biological, behaviour of the impure preparations indicate that more than one, and probably several, different antibacterial substances are produced by the various strains of group C. All the substances are heat-stable, but they differ from the lateritiin group in being insoluble in light petroleum, ether or chloroform.

Group D. Sixteen *Fusarium* strains were inactive against both *Myco. phlei* 1 and *Staph. aureus* in all tests made at frequent intervals up to 28 days of growth. Five of these (*F. culmorum* 2, *F. sambucinum* 3, *F.* 10 and *F.* 88 (from tomato) and *F.* 96 (potato) were not tested further; the rest were tested against *Myco. phlei* 2 with repeat tests of *Myco. phlei* 1 for control. Five strains (*F. dimerium*, *F. caeruleum*, *F.* 95 and *F.* 97 from potato and *F.* 132 from cob-nuts) were inactive against both *Myco. phlei* 1 and 2 but the remaining six,

though again inactive against *Myco. phlei* 1, showed various degrees of activity against *Myco. phlei* 2. Three strains, *F. oxysporum cubense*, *F.* 124 and *F.* 127 (hop canker) were inhibitory only in 1/5 dilution and for a limited period; *F. moniliforme-subglutinans* 2 developed a maximum potency of 1/15. *F. aquaeductum* and *F.* 94 (potato) were inactive until the 12th day when a potency of 1/10 against *Myco. phlei* 2 suddenly developed. The potency of *F.* 94 filtrates remained constant until the 21st day; that of *F. aquaeductum* increased to a maximum of 1/20 on the 20th day, dropping to 1/15 on the 29th day. Since lateritiin and fructigenin are considerably more active against *Myco. phlei* 2 than against *Myco. phlei* 1 (see below), it may be that the antibiotics produced by these six strains of group D are of a similar type to those of group B, but are produced in amounts too small to be detected in tests of the culture fluids against *Myco. phlei* 1. This seems likely where maximum potencies of 1/5 against *Myco. phlei* 2 were obtained, but the case of *F. aquaeductum*, when a culture fluid of 1/20 potency against *Myco. phlei* 2 was inactive against *Myco. phlei* 1, is more difficult to explain. No attempts were made to isolate the antibiotic substances from any of the group D cultures.

Antibacterial activity of pure Fusarium *antibiotics*

In group A two antibiotic substances, namely the pigments javanicin and oxyjavanicin produced by *F. javanicum*, have been isolated in a pure state (Arnstein, *et al.* 1946*a*, *b*).

In group B antibacterial substances have been extracted from the culture fluids of eight strains. A preliminary account of five of these, namely lateritiin I and II, avenacein, sambucinin and fructigenin from strains of *F. lateritium*, *F. avenaceum*, *F. sambucinum* and *F. fructigenum* respectively, has been published (Cook *et al.* 1947). These five substances are so similar in chemical behaviour as to leave no doubt that their biological activity is due to similar structural features, but both their chemical and antibacterial reactions show that they are distinct compounds. The differences in antibacterial behaviour are most marked against the two strains of *Myco. phlei*. Lateritiin I and II and fructigenin are much more effective against *Myco. phlei* 2 than against *Myco. phlei* 1, avenacein is equally effective against both strains and sambucinin is considerably less active against *Myco. phlei* 2. Lateritiin I, lateritiin II and fructigenin are distinguished by the ratios of their activity against *Staph. aureus*, *Strep. pyogenes* and *B. subtilis*. The chemical identity of the three substances isolated from the unidentified *Fusarium* strains *F.* 135, *F.* 136 and *F.* 138 was not fully established, but the similarity of the antibacterial activity of the crude culture fluids, the impure extracts and the pure crystalline antibiotic substances provides strong evidence of their identity with fructigenin. Enniatin, isolated from *Fusarium orthoceras* var. *enniatinum* by Gäumann, Roth, Ettlinger, Plattner & Nager (1947) is very similar, both chemically and in antibacterial activity, to the antibiotics of group B.

Table 2 shows the highest dilutions of the nine *Fusarium* antibiotics which give complete bacteriostasis of five bacterial strains. *Fusarium* antibiotics are

Table 2. *Relative bacteriostatic action of nine* Fusarium *antibiotics against five bacterial strains, the potency against* Mycobacterium phlei 1 *being taken as* 100

Antibiotic	*Myco. phlei* 1	*Myco. phlei* 2	*Staph. aureus*	*Strep. pyogenes*	*B. subtilis*
Javanicin	1/200,000 (100)	50	100	20	100
Lateritiin I	1/200,000 (100)	160	25	20	40
Lateritiin II	1/160,000 (100)	200	25	15	20
Avenacein	1/160,000 (100)	100	12·5	6	3
Sambucinin	1/200,000 (100)	25	20	10	5
Fructigenin	1/200,000 (100)	150	15	25	10
135	1/160,000 (100)	150	25	30	15
137	1/150,000 (100)	133	26	33	13
138	1/160,000 (100)	150	25	30	15

bactericidal as well as bacteriostatic, although in most cases a considerably stronger concentration of the antibiotic is necessary for killing. Table 3 shows the result of an experiment to find the time required for bactericidal action in broth. One drop of a broth culture of *Myco. phlei* 1 was used as inoculum into each broth + antibiotic tube. Subcultures were made immediately and at intervals up to 48 hr. on nutrient agar plates and the colonies counted.

Table 3. *Bactericidal action of four* Fusarium *antibiotics against* Mycobacterium phlei 1 *in broth*

		Percentage of bacteria living after (hr.)			
Antibiotic	Dilution	2	4	24	48
Lateritiin I	1/200,000	70	70	22	0
Lateritiin I	1/100,000	60	46	3	0
Lateritiin II	1/20,000	50	20	3	0
Avenacein	1/20,000	64	50	0	0
Sambucinin	1/20,000	27	27	0	0
—	Control broth	100	100	Large increase	

Growth of the few bacteria that survived for 24 hr. in the lateritiin I and II broths was much delayed, tiny colonies being only just visible after 3 days' incubation. Even the 1/200,000 dilution of lateritiin I, which is the highest dilution of this antibiotic to cause complete bacteriostasis of *Myco. phlei* 1, is markedly bactericidal, though somewhat slower in action than the 1/100,000 dilution.

Effect of blood serum on Fusarium *antibiotics*

Lateritiin I and II, avenacein and sambucinin were tested in broth containing various amounts of horse serum, using *Staph. aureus* as the test organism. The results are given in Table 4. In the presence of serum a higher concentration of antibiotic is required for complete bacteriostasis but, except in the 40 % serum test of lateritiin I, normal growth of *Staph. aureus* occurred only in the same dilution of antibiotic in presence or absence of serum. In the dilutions causing partial inhibition there was a marked decrease in the growth of *Staph. aureus* and the bacteria were strongly agglutinated. *Staph.*

Table 4. *The effect of blood serum on the activity of* Fusarium *antibiotics against* Staphylococcus aureus

Antibiotic	Serum (%)	Dilution of antibiotic giving: Complete inhibition	Partial inhibition	Normal growth
Lateritiin I	—	1/40,000	1/50,000	1/80,000
	10	1/20,000	1/40,000	1/80,000
	20	1/10,000	1/40,000	1/80,000
	25	1/10,000	1/20,000	1/80,000
	40	1/10,000	1/20,000	1/40,000
Lateritiin II	—	1/40,000	—	1/80,000
	10	1/20,000	(No higher dilution tested)	
	25	1/20,000	1/40,000	1/80,000
Avenacein	—	1/10,000	1/20,000	1/40,000
	10	—	1/10,000	—
	25	1/5,000	1/20,000	1/40,000
Sambucinin	—	1/40,000	—	1/80,000
	10	1/20,000	1/40,000	—
	25	1/10,000	1/20,000 to 1/40,000	1/80,000

aureus grew normally in control tubes of broth containing 25 or 40 % serum, and there was considerably heavier growth in these tubes than in the control tubes without serum. Therefore in comparing the activity of the antibiotics in the presence and absence of serum allowance should be made for the fact that the serum broth was a decidedly more favourable medium for the growth of *Staph. aureus* than the plain nutrient broth.

Destruction of lateritiin in cultures

Cook *et al.* (1947) suggested that the *Fusarium* antibiotic substances are normal but usually transient products of metabolism which accumulate in the culture fluids in detectable quantities only under abnormal cultural conditions. If this be so, the absence of activity in the culture fluids of some *Fusarium* strains might be due, not to failure to produce antibiotic substances, but to their rapid conversion into other compounds by the continued growth of the culture. This view is supported by the fact that the inactive strains were nearly always the most vigorous in growth. The following experiments were designed to test this suggestion.

Experiment 1. A solution of pure lateritiin in Czapek-Dox medium, having a potency of 1/10 against *Myco. phlei* 1, was divided into four equal parts, one of which was left uninoculated for control. The other three parts were inoculated respectively with: *F.* 97, a very vigorously growing strain but producing no antibiotic; *F. lateritium* 1, the strain from which lateritiin was produced; a degenerate culture of *F. lateritium* which gave a very poor gelatinous growth. The cultures were incubated at 25° and tested against *Myco. phlei* 1 after 5, 7 and 10 days. The culture of *F.* 97 was active at 1/5 but not at 1/10 on the 5th day and was inactive on the 10th day when the two *F. lateritium* cultures

and the control were still active in 1/10 dilution. Thus the lateritiin was destroyed only by the vigorously growing strain *F.* 97.

Experiment 2. *F. lateritium* was grown on the standard Eupeptone medium until the culture fluid had developed a potency of 1/10 against *Myco. phlei* 1. The fluid was then filtered to remove the mycelium, heated to 100° for 10 min. to sterilize, and dispensed into three sterile flasks. The first flask was kept as control, the second was inoculated with the parent culture of *F. lateritium* and the third with the vigorous grower *F.* 97. The flasks were incubated at 25°. The difference in growth of the two *Fusarium* strains was very marked. Growth was visible in the *F.* 97 flask after 24 hr. and the mycelium had spread throughout the medium in 48 hr. *F. lateritium*, however, showed no sign of growth in 24 hr. and there was only a very slight development of mycelium round the piece of inoculum on the 3rd day. Thus an old culture filtrate of *F. lateritium* is a good medium for the growth of the *F.* 97 strain which does not produce antibiotic, but a very poor medium for *F. lateritium*. Tests at 4 and 7 days showed destruction of lateritiin by *F.* 97 but not by *F. lateritium*. The cultures were left incubating until the 25th day, when they were retested; the control uninoculated fluid had its original potency 1/10 dilution against *Myco. phlei* 1, the *F. lateritium* fluid was still effective in 1/5 dilution, but all traces of activity had disappeared from the *F.* 97 culture.

Experiment 3. Strain *F.* 97 was seeded into tubes containing pure lateritiin in (*a*) distilled water, and (*b*) a weak (1/5 usual) Czapek-Dox medium; both solutions were initially active in 1/20 dilution against *Myco. phlei* 1. After 3 days' incubation at 25° (*a*) was only partially active against *Myco. phlei* 1 in 1/5 dilution. The destruction of lateritiin in solution (*b*) took longer, probably because there was an alternative supply of nutrient material available, but both solutions were quite inactive on the 10th day. The control tubes of each solution were unaltered in potency.

Experiment 4. *F. lateritium* and the non-producing strain *F.* 97 were grown together in mixed culture and, as control, an equal quantity of culture medium was inoculated with pure *F. lateritium*. Both strains grew well in the mixed culture, which consisted of the pure white felt of *F. lateritium* and the deep red felt of *F.* 97. The pure culture of *F. lateritium* developed normally, i.e. had a potency of 1/10 on the 4th day, rising to 1/20 on the 11th with a fall to 1/10 again on the 21st and 23rd days. The mixed culture was active in 1/5 only on the 4th day and did not at any time reach a potency of more than 1/10, which fell again to 1/5 on the 23rd day. These experiments, which indicate that a vigorously growing *Fusarium* strain which does not produce antibiotic is capable of inactivating lateritiin, support the theory that *Fusarium* antibiotic substances are normal products of the fungal metabolism.

Antifungal action of Fusarium *species*

The *Fusarium* strains tested had little or no antifungal action: sometimes there was slight mutual antagonism between species grown on the same culture-plate but generally no inhibitory action was observed. Arnstein *et al.* (1946*b*)

noted that javanicin is almost without antifungal activity. Lateritiin, except for causing a slight initial lag in growth of *Botrytis cinerea* and *Verticillium* sp., also showed no antifungal activity.

The author wishes to thank all those to whom she is indebted for *Fusarium* cultures, in particular Mr M. H. Moore (who sent the *F. lateritium* culture from which lateritiin was obtained) and Miss Joan Moore. The chemical work was carried out in the organic chemistry department of the Imperial College by Drs H. R. V. Arnstein, S. F. Cox and T. H. Farmer under the direction of Dr A. H. Cook. Thanks are due to the Medical Research Council for financial assistance.

REFERENCES

Arnstein, H. R. V., Cook, A. H. & Lacey, M. S. (1946*a*). An antibiotic pigment from *Fusarium javanicum*. *Nature, Lond.*, **157**, 333.

Arnstein, H. R. V., Cook, A. H. & Lacey, M. S. (1946*b*). Production of antibiotics by fungi. Part II. Production by *Fusarium javanicum* and other *Fusaria*. *Brit. J. exp. Path.* **27**, 349.

Cook, A. H., Cox, S. F., Farmer, T. H. & Lacey, M. S. (1947). Production of antibiotics by *Fusaria*. *Nature, Lond.*, **160**, 31.

Gäumann, E., Roth, S., Ettlinger, L., Plattner, P. A. & Nager, U. (1947). Enniatin, ein neues gegen Mycobakterien wirksames Antibioticum. *Experientia*, **3**, 202.

(*Received* 14 *May* 1949)

Factors Affecting the Fruiting of *Chaetomium* Species

By S. N. BASU and R. G. BOSE
The Microbiology Department, Indian Jute Mills Association Research Institute, Calcutta

SUMMARY: The effect of temperature and some chemical substances on the fruiting of strains of nine species of *Chaetomium* was studied. The optimum temperature for fruiting was generally lower than that for growth. Micro-nutrients of the nature of growth factors and starvation with respect to nutrients may both stimulate fruiting, whereas staling inhibits it, the ultimate balance between these effects determining the fruiting behaviour of a given species. These factors had no significant effect on the final amount of the vegetative growth within the conditions of the experiments.

In a previous paper (Buston & Basu, 1948) some experiments with a strain of *Chaetomium globosum* Kunze (Lab. ref. no. *79*) were reported wherein it was found that the fungus did not fruit unless the concentration of soluble sugar in the medium was very low, although this condition did not necessarily engender perithecia under all circumstances. A water extract of jute had considerable effect on fruiting and on rate of growth. In the experiments reported here, the effect of these and other factors on the fruiting of strains of several species of *Chaetomium* was studied and the nature of the stimuli needed to induce the sexual cycle in them investigated.

MATERIALS AND METHODS

The ten strains used, representing nine different species, and their laboratory reference numbers were as follows: *108*, *C. cancroideum* Tschudy; *109*, *C. ochraceum* Tschudy; *110*, *C. cochliodes* Palliser; *111*, *C. dolichotrichum* Ames; *112*, *C. funicola* Cooke; *115*, *Chaetomium* sp.; *116*, *C. elatum* Kunze & Schmidt; *116·1*, *C. elatum* Kunze & Schmidt; *79*, *C. globosum* Kunze; *75*, *C. indicum* Corda.

All but the last three strains were identified and supplied by the Commonwealth Mycological Institute at Kew, where the identity of *79* and *75* was also checked. No. *115* is recorded at Kew as *C. globosum*, but we found its perithecia nearly 50 % bigger on the average than those of *C. globosum*. All stock cultures were grown on malt extract agar slants and stored in the refrigerator. Inoculation was made with a loopful of suspension of ascospores prepared by crushing a few perithecia in a drop of sterile distilled water.

The basal medium contained: $NaNO_3$, 2 g.; K_2HPO_4, 1 g.; KCl, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; in 1 l. water. Liquid media were used in 10 ml. quantities in 100 ml. Pyrex conical flasks, solid media (with 15 g./l. agar) were placed in 20 ml. quantities in 9 cm. Petri dishes. Media were sterilized at 10 lb. for 20 min. All other variable details are given in the tables. The numbers 1, 2, 3 and 4 were used to denote increasing intensity of fruiting.

RESULTS

Temperature

All the strains, except *79* and *75*, fruited better at 22° than at 30°, although the rate of colony growth was faster at 30° for five (Table 1).

Table 1. *Effect of temperature on fruiting*

Growth on agar medium; glucose 1 %; incubation at 22° or 30° as shown. Increasing intensity of fruiting indicated by numbers 0 to 4.

	22°	30°	22°	30°	22°	30°	22°	30°
	Period for appearance of perithecia (days)		Intensity of fruiting at 28 days		Colony diameter (mm.)			
					At onset of fruiting		At 21 days	
Strain								
108	15	15	3	2	60	82	68	87
109	21	>28	2	0	94	—	94	94
110	11	13	4	2	78	>95	>95	>95
111	9	9	4	2	45	65	85	91
112	11	9	3	2	42	40	75	79
115	24	>28	1	0	52	—	49	73
116	17	>28	2	0	>95	—	>95	94
116·1	17	>28	1	0	>95	—	>95	85
79	21	11	1	3	65	38	65	71
75	>28	15	0	2	—	>95	79	>95

Carbohydrates as the carbon source

Type of carbohydrate. Table 2 shows that cellulose (Whatman filter paper no. 42), starch, and maltose give the biggest sum totals of the figures representing intensity of fruiting; these were also the only carbohydrates that stimulated fruiting of all the strains tested in agar medium. Sucrose and dextrin were fairly good C sources, and stimulated maximum fruiting of three and four strains, respectively. Glucose, fructose and arabinose seldom gave a good perithecial crop, many strains producing no perithecia at all, although vegetative growth was often as good as on any of the other compounds tested.

The agar in the media no doubt influenced the results favourably, since perithecia appeared later on a cellulose medium without agar (filter paper suspended in the basal medium) than on cellulose-agar medium; the ultimate perithecial density was also smaller and three strains, *108*, *109* and *116·1*, did not fruit at all on the agarless medium.

Concentration of carbohydrate. The secondary influence of growth factors may also have been responsible for the excellent results given by maltose (a Merck product was used) which has been shown to be liable to contamination with aneurin (Schopfer, 1934). However, the polysaccharides proved better than the other sugars in producing perithecia. That this might be due to a low soluble sugar concentration in the medium is suggested by the results of the experiments recorded in Tables 3 and 4. These showed that a lowering of the initial glucose concentration in the medium favoured fruiting and that little glucose was left when perithecia appeared, although exhaustion of glucose by

Table 2. *Effect of various carbohydrates*

Agar medium. All carbohydrates 3 % concentration except cellulose 1 %. Incubation at 22°. Increasing intensity of fruiting indicated by numbers 0 to 4; * = immature perithecia.

Strain	Period for appearance of perithecia (days)								Intensity of fruiting at 21 days								Colony diameter (mm.) at 14 days							
	Arabinose	Glucose	Fructose	Sucrose	Maltose	Dextrin	Starch	Cellulose	Arabinose	Glucose	Fructose	Sucrose	Maltose	Dextrin	Starch	Cellulose	Arabinose	Glucose	Fructose	Sucrose	Maltose	Dextrin	Starch	Cellulose
108	>21	>21	17	14	14	>21	21	14	0	0	3	4	2	0	1	2	52	>95	45	65	>95	88	>95	80
109	11	>21	17	11	8	11	8	6	1	0	1*	3	3	3	4	3	58	>95	>95	>95	>95	>95	83	>95
110	8	8	8	14	8	11	6	6	3	1	2	2	2	4	4	3	>95	>95	>95	90	>95	>95	80	>95
111	8	11	8	8	8	6	6	6	3	4	4	4	4	4	4	4	37	60	80	85	72	62	61	80
112	>21	8	6	6	8	8	6	6	0	2	3	4	4	4	4	2	18	46	32	47	51	45	42	60
115	>21	>21	21	21	6	6	8	6	0	0	1	1	4	2	2	4	72	45	60	38	48	53	50	>95
116	>21	>21	>21	>21	6	11	11	6	0	0	0	0	3	2	2	3	30	90	85	42	>95	88	72	>95
116·1	>21	>21	>21	17	14	14	11	8	0	0	0	2	2	2	2	3	50	>95	85	34	70	90	80	>95
79	8	>21	8	14	11	6	8	6	4	0	1*	2	3	4	4	4	85	58	55	60	50	50	55	>95
75	>21	17	>21	17	11	11	11	11	0	2	0	2	3	3	2	2	35	73	69	>95	>95	78	80	70

itself did not induce fruiting in all cases. Two apparent anomalies are shown by strains *108* and *111*; *108* was indifferent to the changes in initial glucose concentration and *111* sporulated in a relatively large glucose concentration which probably explains the quickness and ease with which it fruited throughout these experiments (see, in particular, Tables 2 and 3).

Table 3. *Effect of varying concentrations of glucose*

Agar medium; incubation at 22°. Increasing intensity of fruiting indicated by numbers 0 to 4.

	Glucose (%)			Glucose (%)			Glucose (%)		
	1·0	0·5	0·25	1·0	0·5	0·25	1·0	0·5	0·25
Strain	Period for appearance of perithecia (days)			Intensity of fruiting at 28 days			Colony diameter (mm.) at 14 days		
108	>28	>28	>28	0	0	0	44	48	60
109	>28	>28	10	0	0	3	50	40	46
110	>28	>28	14	0	0	3	68	45	58
111	19	14	14	4	4	4	70	50	65
112	19	14	14	3	3	2	48	45	53
115	28	24	14	1	1	1	28	25	30
116	>28	26	10	0	2	4	38	44	31
116·1	>28	26	14	0	1	3	70	75	65
79	26	19	14	2	3	4	36	28	40
75	>28	>28	21	0	0	1	55	65	72

Table 4. *The concentration of glucose at onset of fruiting*

Liquid medium; initial glucose concentration and incubation temperatures as shown. Glucose estimated at time of onset of fruiting, or at 30 days (indicated by *).

	Initial glucose 0·1 %; 32°		Initial glucose 1·0 %; 22°	
Strains	Period for appearance of perithecia (days)	Glucose (%) at onset of fruiting, or at 30 days	Period for appearance of perithecia (days)	Glucose (%) at onset of fruiting, or at 30 days
108, 115, 116, 116·1	>30	<0·025*	>30	<0·025*
109	—	—	>30	<0·025*
110	19	<0·025	21	0·026
111	19	<0·025	18	0·054
112	22	<0·025	17	0·032
79	12	<0·025	21	0·032
75	5	<0·027	>30	<0·025*

Nitrogen and phosphorus

Type of nitrogen source. Of the four sources of nitrogen tried (N concentration as in basal medium), peptone (British Drug Houses Ltd., bacteriological) gave the best results, and asparagine and sodium nitrate in that order, the next best (Table 5). Ammonium sulphate, though generally a poor N source, proved surprisingly good for a few strains, including that of *C. globosum* and was the only one of the four N sources which induced fruiting in *108*. However, on the $(NH_4)_2SO_4$ medium prepared with the agar repeatedly washed in changes

Table 5. *Effect of various nitrogen sources*

Growth on agar medium containing 0·5 % glucose; incubation at 22°. Increasing intensity of fruiting indicated by numbers 0 to 4.

Strain	Period for appearance of perithecia (days)					Intensity of fruiting at 21 days					Colony diameter (mm.) at 14 days				
	Sodium nitrate	Asparagine	Peptone	Ammonium sulphate	Ammonium sulphate with washed agar	Sodium nitrate	Asparagine	Peptone	Ammonium sulphate	Ammonium sulphate with washed agar	Sodium nitrate	Asparagine	Peptone	Ammonium sulphate	Ammonium sulphate with washed agar
108	>21	>21	>21	14	19	0	0	0	1	1	75	90	88	43	38
109	14	14	10	17	>21	4	3	4	2	0	75	92	>95	56	30
110	8	8	8	21	19	4	4	4	1	1	70	80	>95	71	45
111	10	10	8	8	9	3	3	4	4	4	76	82	91	67	46
112	8	8	7	11	16	3	3	3	4	2	48	50	71	33	29
115	10	8	8	>21	>21	1	2	4	0	0	45	36	56	25	20
116	8	8	8	>21	>21	2	3	4	0	0	65	93	94	50	25
116·1	8	8	8	>21	>21	4	4	4	0	0	51	90	>95	78	48
79	10	9	8	11	>21	4	4	4	4	0	38	35	65	38	21
75	21	12	10	17	19	1	2	3	1	1	86	>95	>95	54	48

of cold distilled water fruiting was delayed and the colonies smaller in all cases.

Tschudy (1937) reported that peptone was a poor nutrient for *Chaetomium* species and suppressed the normal development of perithecia. This is in direct conflict with our results, but in Tschudy's experiments washed and unwashed agar gave substantially the same results.

Table 6. *Effect of nitrogen- and phosphorus-starvation*

Growth on agar medium containing 1 % glucose; incubation at 22°. Increasing intensity of fruiting indicated by numbers 0 to 4.

	Medium			Medium			Medium		
	Complete	No N	No P	Complete	No N	No P	Complete	No N	No P
Strain	Period for appearance of perithecia (days)			Intensity of fruiting at 28 days			Colony diameter (mm.) at 14 days		
108	>28	>28	>28	0	0	0	44	44	62
109	>28	>28	28	0	0	1	50	65	65
110	>28	>28	27	0	0	1	68	70	85
111	19	19	24	4	3	2	70	75	90
112	19	>28	19	3	0	3	48	58	62
115	28	10	24	1	1	1	28	35	39
116	>28	>28	>28	0	0	0	38	70	75
116·1	>28	>28	>28	0	0	0	70	72	73
79	26	14	14	2	3	3	36	75	>95
75	>28	21	21	0	1	1	55	88	72

Nitrogen and phosphorus starvation. Table 6 shows that fruiting of some strains was stimulated on media without K_2HPO_4 and $NaNO_3$ respectively, the effect being rather more marked in the absence of P than of N. No. *79* responded most strikingly. At the other extreme, *111* and *112* were unaffected and fruiting actually seemed to suffer in the absence of P or N. These two strains also showed the smallest response to glucose starvation (Table 3). The increased rate of spread of the colonies of some strains was of course a starvation phenomenon and the colonies were thin.

Effect of other medium constituents

Crude extracts. Five g. of clean jute fibre (*Corchorus capsularis*) were boiled in excess of distilled water for 30 min., the liquor concentrated on the water bath to 100 ml. and filtered. Suitable amounts of this extract (to give the equivalent of the extract from 1·5 g. jute/100 ml. of medium) were added to 5 ml. of the double strength medium, and the volume adjusted with distilled water. This extract was first compared as a source of growth substances with a solution of the ash from jute extract prepared by evaporation and ignition of the extract and solution of the residue in acidified distilled water.

The fruiting of strains *110*, *111*, *112*, *79* and *75* was distinctly improved by extracts of jute and agar, the agar having a relatively weaker action (Table 7); final mycelial weights did not vary significantly. On the other hand, malt extract (40 and 4 mg./100 ml.) and yeast extract (Marmite, 2·5 and 0·25 mg./

100 ml.) had a moderate effect on *111* and *116* only and a doubtful effect on *109* and *110*; *79* and *75* were unaffected. The action of jute extract on *111*, *112*, *79* and *75* can be partly explained by its inorganic contents; however, the fact that this latter action was weaker than that of whole jute extract and the indifference of *110* and *115* towards jute extract ash, though not to the whole extract, suggest that both trace elements and organic growth factors may have

Table 7. *Effect of jute extract, jute extract ash, and agar extract*

Liquid medium. Glucose 0·3 %. Extract of 1·5 g. agar or jute or ash from equivalent amount per 100 ml. Incubation at 27°.

Strain	Control	Jute extract	Jute extract ash	Agar extract
	Period for appearance of perithecia (days)			
108, 109, 115, 116, 116·1	>24	>24	>24	>24
110	16	7	16	12
111	13	9	10	10
112	>24	10	13	15
79	9	6	7	7
75	>24	7	13	12

a favourable effect on the fruiting of *Chaetomium*. Several metallic ions and pure essential metabolites known to stimulate the growth of other fungi were therefore tested.

Trace elements and essential metabolites. $FeSO_4.7H_2O$, $ZnSO_4.7H_2O$, and $MnSO_4.H_2O$ were tested in $1/10^5$ dilution, $CuSO_4.5H_2O$, $Co(NO_3)_2.6H_2O$ and $Na_3PO_4.12MoO_3$ in $1/10^6$ dilution. Biotin alone, and a mixture of aneurin, riboflavin, pyridoxin, Ca pantothenate and nicotinic acid, all at 0·1 μg./10 ml., were tested separately in a liquid medium containing 0·5 % glucose. During 28 days at 22°, no stimulation of growth or fruiting was observed in any strain except *79* which was weakly stimulated by Mn, biotin and the vitamin mixture.

DISCUSSION

Although the formation of perithecia in all the strains tested, with the possible exception of *108*, was stimulated by a low glucose concentration, the fact that some did not fruit even when the sugar was exhausted suggests that one or more staling products that inhibit perithecia formation but not growth may play an important part. The fruiting mechanism may be affected by the staling product in different degrees in different species so that they produce perithecia with varying ease on a medium with a given glucose concentration. The higher the concentration of glucose the greater may be the accumulation of this hypothetical inhibitor of perithecial formation, a point being reached for each species when perithecia are completely suppressed. As regards the behaviour of the different carbohydrates, though it is possible that they produce different amounts of this 'inhibitor' (glucose apparently inducing the highest yields), it is more likely that a scarcity of nutrients in itself exerts a positive and favourable

influence on fruiting. This view is supported not only by the superior performance of the polysaccharide media, which can contain only a relatively small amount of soluble sugar even at the initial stages of growth, but also by the reaction of some species towards P and N starvation; for example, of all the strains, *111* and *112* showed the smallest and *79* the largest response to both glucose and mineral starvation, which may mean that they are respectively the least and the most sensitive towards a deficiency in nutrients (Tables 3 and 6). On the other hand, a few strains, like *116*, responded to low glucose but not to P or N starvation; here staling may be the more important factor.

Staling would also explain the temperature effect observed. With most of the strains tested (Table 1), vegetative growth was more rapid at 30° than at 22°, but the reverse was the case with fruiting, which suggests greater staling as a consequence of increased metabolic activity at the higher temperature. The apparent anomaly of *79* and *75*, which fruit better at 30°, is probably due to their optimum temperatures for vegetative growth being higher than those of the others, evidence for which was, in fact, found in experiments not reported here. The existence of different temperature optima for growth and fruiting in *Chaetomium* is interesting since it is generally believed that the optimum sporulation range in fungi, though narrower, usually lies within the optimum growth range. However, Hawker (1947) found that although the sterile strains of the Ascomycete *Melanospora destruens* grew best at 18° or 20°, the fertile strains developed best at 37°. The *Chaetomium* species as a rule showed the reverse phenomenon, the perithecial optimum being the lower.

That the stimulating action of agar was not, at least wholly, the physical effect of a solid medium was shown by the good response, of all but those five strains which were consistently the most resistant towards fruiting (namely *108*, *109*, *115*, *116* and *116·1*), to a liquid medium containing a water-extract of agar and also by the decreased growth and fruiting of most strains on a medium solidified with washed agar. The good effect of agar seems to be due to its overcoming the staling effect, since agar media gave perithecia quicker than liquid media with the same initial glucose concentration, and also supported perithecia formation with larger amounts of initial glucose. Jute extract seemed to exert the same kind of effect as agar extract, but malt and yeast extracts—known to be potent sources of growth substances—showed at best a feeble effect (except perhaps on *116*) and also did not influence the strains affected most by jute and agar extracts. This suggests a certain specificity in the fruiting stimuli, apart from specificity in sensitivity to staling, among the strains examined.

From the nature and the effective concentrations of jute and agar extracts the stimulating action may possibly be due to compounds such as certain essential metabolites or trace elements. Although no comparable stimulation was shown by the essential metabolites tested and Hawker's (1942) observation that *C. cochliodes* fruited more freely with the addition of aneurin could not be confirmed with the present strain, it remains probable that other substances, or other concentrations or combinations of those we tested would approximate the natural extracts more closely. We are investigating this point.

The fact that, among all the carbohydrates tried as C source, glucose was the least effective in perithecia production is striking, since the three polysaccharides (dextrin, starch, cellulose) that were most effective are all composed of glucose units and are presumably hydrolysed to glucose or phosphorolysed to glucose-1-phosphate as the first step in glycolysis. Nord & Mull (1945), however, pointed out, on the basis of experience with *Fusarium lini* and *Chaetomium funicola*, that the classical mode of pyruvate formation may not be universal and that even non-phosphorylating glycolysis is possible.

In view of the efficacy of peptone as a N source observed by us, Tschudy's (1937) observation that it inhibits perithecia formation in *Chaetomium* remains unconfirmed.

It is evident from the tables that there is no critical degree of growth representing a state of maturity of the fungus which must be reached before perithecia appear. Nowhere was there relation between rate or amount of growth and fruiting. Moreover, the different strains vary in their efficiency of producing cell-substance. The amount of mycelium formed from 50 mg. glucose varied from *c.* 10 mg. in *108* to 20 mg. in *110* and *116·1*.

The strains were fairly consistent in behaviour throughout these experiments and are divisible into two broad groups, one fruiting with difficulty (type example, *108*), the other fruiting more freely (type example, *111*). However, there was considerable interspecies variation, and even the two strains of *C. elatum* (*116* and *116·1*) showed some differences. Sometimes the same strain varied in repeated experiments.

The authors wish to thank the Committee of the Indian Jute Mills Association Research Institute for permission to publish the results described.

REFERENCES

Buston, H. W. & Basu, S. N. (1948). Some factors affecting the growth and sporulation of *Chaetomium globosum* and *Memnoniella echinata*. *J. gen. Microbiol.* **2**, 162.

Hawker, L. E. (1942). The effect of vitamin B_1 on the concentration of glucose optimal for the fruiting of certain fungi. *Ann. Bot., Lond.*, **6** (N.S.), 631.

Hawker, L. E. (1947). Effect of temperature and storage on the rate of loss of fertility of stock cultures of *Melanospora destruens* Shear. *Nature, Lond.*, **159**, 136.

Nord, F. F. & Mull, R. P. (1945). Recent progress in the biochemistry of *Fusaria*. *Advances in Enzymology*, **5**, 165.

Schopfer, W. H. (1934). Les vitamins B comme hormones de croissance chex un micro-organisme. *Arch. Mikrobiol.* **5**, 511.

Tschudy, R. H. (1937). Experimental morphology of some species of *Chaetomium*. II. Reactions of species of *Chaetomium* under various conditions of cultivation. *Amer. J. Bot.* **24**, 657.

(*Received* 2 *June* 1949)

Some Observations on a Streptomycin-dependent Strain of *Staphylococcus aureus*

By G. E. FOLEY and H. SHWACHMAN

The Division of Laboratories and Research, The Children's Medical Centre, and the Department of Pathology and Pediatrics, Harvard Medical School, Boston, Massachusetts

SUMMARY: Variants whose growth was either dependent upon or enhanced by streptomycin appeared spontaneously during a sensitivity titration of a strain of *Staphylococcus aureus* isolated from a nose and throat culture. In addition to these variants, multiple single colony strains from the original culture exhibited a wide range of susceptibility to streptomycin. The variability after exposure to streptomycin in the laboratory was no greater than that exhibited by the original isolates.

The mechanism by which these variants utilize streptomycin is not yet known. Streptomycin inactivated by semicarbazide or L-cysteine did not support or enhance growth. The acquisition of the ability to utilize streptomycin had no measurable effect on the other biochemical characteristics investigated.

When large inocula of the dependent variant were planted on streptomycin-free agar, a few colonies appeared after prolonged incubation. The cells of such colonies underwent a profound morphologic change not unlike the production of 'large bodies' which occurs in several species of bacilli. The progeny of such colonies were of normal morphology and either resistant or susceptible to streptomycin but were no longer dependent on it.

Survival of the variants in chick embryos, except in those instances where mutation occurred, was dependent upon the presence of streptomycin. These variants were mouse avirulent, even in the presence of hog mucin in addition to streptomycin.

The purpose of this paper is to describe a strain of *Staphylococcus aureus* in which 'streptomycin-dependent' and 'streptomycin-enhanced' variants appeared spontaneously during a routine streptomycin sensitivity titration.

Origin of variants

The strain of *Staph. aureus* was isolated from the nasopharynx of a patient with pancreatic fibrosis who had not received streptomycin therapy before the isolation of the culture. When examined for streptomycin sensitivity by a tube-titration method (Price, Nielsen & Welch, 1946), growth was obtained after 24 hr. at 37° only in the streptomycin-free control and in those tubes containing 0·03125 and 0·0625 mg. streptomycin sulphate per ml. The titration was repeated, with essentially similar results, i.e. some growth in the streptomycin-free control tube and in the tube containing 0·0625 mg. streptomycin sulphate per ml. Serial subculture from the streptomycin-free control and the tube containing 0·0625 mg. streptomycin sulphate per ml. to tryptic digest agar with and without streptomycin resulted in the isolation of two distinct variants, one almost completely dependent upon streptomycin for growth ('D' variant), and the other ('E' variant) enhanced in growth by streptomycin.

Sensitivity of single colony strains

Multiple single colony fishings were made from the original blood agar plate to tryptic digest broth and to tryptic digest agar, the latter with and without streptomycin sulphate (0·0625 mg./ml.). Eleven of fifteen such isolates proved on subculture to be non-dependent and non-enhanced, while a streptomycin-dependent variant was derived from the progeny of one and streptomycin-enhanced variants were derived from the progeny of three single colonies.

The broth cultures of the eleven non-dependent, non-enhanced single colony isolates were plated on tryptic digest agar and a number of single colony isolations again made in broth, incubated, plated and in turn fished to broth. By this procedure, forty-four single colony strains were derived from the eleven original single colony strains. The streptomycin sensitivity of each was determined by tube titration (Table 1). The single colonies isolated from the original blood agar plate varied markedly in their sensitivity to streptomycin. The broth culture progeny of these single colonies, when fished from tryptic digest plates, again showed striking dissimilarity to their parent colonies as regards streptomycin sensitivity through the second and third subcultures.

Table 1. *Streptomycin sensitivities of single colony isolates of* Staphylococcus aureus *as indicated by a minimal inhibitory dose of streptomycin sulphate* (M.I.D.)

	First subculture original single colony isolates		Second subculture		Third subculture	
	No. isolates	M.I.D. (mg./ml.)	No. isolates	M.I.D. (mg./ml.)	No. isolates	M.I.D. (mg./ml.)
	1	1·0	3	0·125	1	0·03125
			6	0·0625	3	0·016
			1	0·03125	1	0·008
	3	0·5				
	4	0·25	3	0·25	1	2·0
			2	0·125	4	1·0
			1	0·0625		
			1	0·03125		
			1	0·016		
			1	0·008		
	1	0·125				
	1	0·0625				
	1	0·016	2	0·25	5	0·016
			3	0·125		
			2	0·0625		
			3	0·03125		
Total	11		29		15	(55)

Streptomycin sensitivity titrations also were done on single colony isolates derived from a culture which had grown in the presence of a known concentration of streptomycin sulphate. The last tubes in which growth occurred in two of the streptomycin titrations listed in Table 1 were plated on tryptic digest agar, incubated and single colony subcultures made in tryptic digest broth. The last tubes showing growth in streptomycin sensitivity titrations made on

these isolates were then treated in a similar manner. The data obtained from the study of fifty-six single colony isolates derived from the original in this manner are summarized in Table 2.

Table 2. *Streptomycin sensitivities of single colony isolates of* Staphylococcus aureus *after exposure to streptomycin at different concentrations* (*mg./ml.*)

Single colony isolates from culture exposed to streptomycin (mg./ml.)	First subculture		Second subculture		Third subculture	
	No. isolates	Grew in* (mg./ml.)	No. isolates	Grew in (mg./ml.)	No. isolates	Grew in (mg./ml.)
0·25	1	2·0	5	1·0	1	1·0
(inhibited by 0·5)†			2	0·0625	2	0·25
			1	0·016	1	0·125
			1	0·008	1	0·0625
0·0625	4	2·0	6	0·03125	5	0·016
(inhibited by 0·125)†	1	2·0	2	0·016		
	3	1·0	1	0·008		
			1	0·004		
	3	0·5	4	0·03125	1	0·125
			6	0·016	2	0·03125
					2	0·016
Total	12		29		15	(56)

* M.I.D. would be twice these values.
† Derived from isolates listed in Table 1.

It is apparent that exposure to a given concentration of streptomycin did not fix the sensitivity of this strain at or above that concentration, since there was as much fluctuation in streptomycin sensitivity among these single colony isolates as was observed among those derived from the original blood agar plate which had not been exposed to streptomycin. These isolates were uniformly resistant to penicillin (more than 2·5 μg./ml.) but sensitive to both bacitracin (0·5 μg.) (Johnson, Anker & Meleney, 1945) and aureomycin (0·5 μg.) (Duggar, 1948). Penicillinase (Bondi & Dietz, 1944) was produced by all isolates tested.

A second nasopharyngeal culture was obtained from the same patient 2 months later, after 3 weeks of aerosol therapy which consisted of three or four daily inhalations of penicillin (100,000 units) and streptomycin (0·2 mg.). Similar studies on this strain of *Staphylococcus aureus* gave essentially the same results.

Effect of streptomycin on the D and E variants

The D variant grew in media containing as little as 0·00005 mg. or as much as 50 mg. streptomycin sulphate per ml. Growth was sparse and sporadic in concentrations less than 0·00005 mg./ml., and concentrations of more than 50 mg./ml. were inhibitory. The D variant, when planted heavily in streptomycin-free media and incubated for relatively long periods of time, usually yielded a slight amount of growth, for example, a streptomycin-free tryptic digest plate streaked with a 2 mm. loopful of colonial growth usually grew ten or twenty colonies after 48–72 hr. incubation at 37°. (Similar inocula streaked

on streptomycin tryptic digest agar resulted in a heavy, confluent growth after 24 hr. at 37°.) The progeny of the colonies from the streptomycin-free plate were streptomycin-sensitive or resistant, but none was streptomycin-dependent.

Similar experiments done with the E variant yielded comparable results. The range of streptomycin concentrations which enhanced growth and the inhibitory dose were essentially the same as those supporting and inhibiting the growth of the D variant

In vivo *experiments*

The dependence of the D variant on streptomycin raised the question as to whether or not this strain could survive *in vivo* in the absence of streptomycin.

Ten-day chick embryos were prepared by the usual window method and treated with 2·5 mg. streptomycin sulphate on the chorioallantoic membrane. Similar numbers of treated and control embryos were then inoculated on the chorioallantoic membrane with 0·1 ml. (*c.* $4{\cdot}5 \times 10^6$ cells) of a saline suspension of the D variant prepared from a tryptic digest streptomycin agar plate. The eggs were incubated at 37°, examined daily by smear and plated at 48 hr. intervals on tryptic digest agar with and without streptomycin to determine the viability of the staphylococci seen by smear.

Although different experiments showed some individual variation, the overall results were similar to those summarized in Table 3. Streptomycin appeared to be essential for the survival and growth of the D variant in 10-day chick embryos, except in those instances in which a non-dependent mutant appeared after prolonged incubation. It is interesting to note that the D variant gave rise to non-dependent mutants in the chick embryo as well as in artificial media.

Table 3. *Effect of streptomycin on survival of the D variant of* Staphylococcus aureus *in chick embryos*

	Streptomycin, 2·5 mg.		No streptomycin	
Incubation period (hr.)	Gram stain showed	Cultures	Gram stain showed	Cultures
24	Heavy growth	—	Few cells, intracellular	—
48	Heavy growth	STDA 4+ TDA 0	Few cells, intracellular	STDA 0 TDA 0
72	Heavy growth	—	Few cells, intracellular	—
96	Heavy growth	STDA 4+ TDA 0	Few cells, intracellular	STDA 0 TDA 1+

TDA = tryptic digest agar.
STDA = tryptic digest agar + streptomycin sulphate, 0·1 mg./ml.
4+ = more than 100 colonies.
1+ = less than 20 colonies.

The D and E variants were avirulent for mice, even when injected intraperitoneally with hog mucin as well as streptomycin. The non-dependent, non-enhanced isolates of the strain from which these were derived were also mouse avirulent.

Substitution for streptomycin

Dihydrostreptomycin sulphate (Bartz, Controulis, Crooks & Rebstock, 1946) could be substituted for streptomycin with either the D or E variant. Streptomycin sulphate inactivated by semicarbazide hydrochloride (Rake & Donovick, 1946) or by L-cysteine hydrochloride (Denkelwater, Cook & Tishler, 1945) did not support growth of the D variant or enhance the growth of the E variant, when tested in tryptic digest broth and agar or sodium thiolacetate broth.

Ammonium, ferric, or adenine sulphate, sodium thiolacetate, L-cystine, L-cysteine, guanidine carbonate, guanine hydrochloride, 2, 4, 5-triamino-6-hydroxypyrimidine and *i*-inositol, singly or in their various combinations in concentrations of 30, 60 and 120 μg./ml. medium could not be substituted for streptomycin with either the D or E variant. Similarly, the addition of trace elements, Fe, Mg, Mn, Cu, Zn, and Co (as sulphate or chloride) or vitamins, calcium pantothenate, aneurin hydrochloride, nicotinic acid, riboflavin, pyridoxin, folic acid and biotin, in concentrations of as much as 10 μg./ml., did not support growth of the D variant or enhance growth of the E variant. Crystalline penicillin G, bacitracin sulphate or aureomycin could not be substituted for streptomycin in similar concentrations. Bacitracin and aureomycin, when titrated in the presence of 0·1 mg./ml. of streptomycin, inhibited both the D and E variants.

Biochemical studies

The biochemical characteristics of the D and E variants were determined in the presence of 0·1 mg. streptomycin sulphate per ml. of the various media. The non-dependent, non-enhanced variants were tested in the same media without streptomycin for comparison. All strains fermented glucose, maltose, mannitol, lactose and sucrose (but not xylose or dulcitol), liquefied gelatin and split urea.

It has been observed that penicillin and streptomycin delay or inhibit the formation of plasma clots by staphylocoagulase (Agnew, Kaplan & Spink, 1947). The ability of the D and E variants to coagulate sterile human plasma in the presence of streptomycin was determined by the addition of 0·5 ml. of plasma to 0·5 ml. of tryptic digest broth containing concentrations of streptomycin sulphate such that the final concentration after the addition of plasma ranged from 25 mg. to 0·00005 mg./ml. Each tube was inoculated with a loopful of an 18 hr. tryptic digest broth culture of the strain to be tested. Several non-dependent, non-enhanced variants were tested in the same manner, as well as in streptomycin-free media for comparison. The tests were incubated and read at intervals (Table 4). Streptomycin in the concentrations used did not interfere with the coagulase activity of the D and E variants. Both produced firm plasma clots after 2–4 hr. incubation. Further incubation (24–72 hr.) of the coagulated plasma resulted in lysis of the clot.

The results of coagulase tests with non-dependent, non-enhanced isolates in streptomycin-free media were the same as those described for the D and E variants. However, in the presence of sub-inhibitory concentrations of strepto-

mycin, clot formation was delayed, although not entirely inhibited; incubation for 12–24 hr. was required for the formation of a plasma clot (Table 4).

Table 4. *Effect of streptomycin on coagulase activity of various strains of* Staphylococcus aureus

Strain	Streptomycin sulphate (mg./ml.)	Period of incubation at 37° (hr.) 1	2	4	12	24	48
		Character of clot*					
Dependent	25–0·00005	0	2+	4+	4+		Lysed
	0	No growth					
Enhanced	25–0·00005	0	2+	4+	4+	—	Lysed
	0	0	0	2+	4+	4+	Lysed
Normal†	0·5–0·125	0	0	0	2+	4+	Lysed
	0	0	2+	4+	4+		Lysed

* Barely visible clot = 1, to firm clot = 4+.
† Minimum inhibitory dose for this strain was 1·0 mg./ml.

Morphology

The colonial morphology of the original strain was that of the usual *Staph. aureus*; pigmentation was marked, and colonies were of uniform size and were non-haemolytic. The colonies of the D and E variants on tryptic digest agar containing streptomycin tended to be somewhat smaller and less pigmented than either those of normal isolates or of the E variant on streptomycin-free agar.

The cells of the normal isolates and the E variant were of characteristic staphylococcal morphology. However, smears of the D variant always contained a few large, swollen cocci (Pl. 1, fig. 1). When transplanted to streptomycin-free tryptic digest agar and incubated 48–72 hr., a few colonies grew from heavy inocula. These were at first 'pin-point', but eventually matured into typical, deeply pigmented colonies 2–3 mm. in diameter, composed entirely of large, swollen, yeast-like cocci not unlike the 'large bodies' produced by several species of bacillary cells (Dienes, 1939–47; Dienes & Smith, 1944; Pl. 1, fig. 2).

When suitable agar block preparations were made and stained with methylene blue (Dienes & Smith, 1944) these colonies of large cells could be seen developing on the streak completely surrounded or submerged by cells of normal morphology which appeared to be dead, since they did not develop into colonies (Pl. 1, fig. 3).

When a colony of these large cells was transplanted, and followed at frequent intervals by microscopic study of agar block preparations, the large cells were observed to divide by what appeared to be simple fission, eventually giving rise to normal staphylococci which were streptomycin susceptible or resistant, but no longer streptomycin dependent, and were otherwise identical with the parent culture (Pl. 1, figs. 4–7).

DISCUSSION

Studies on the origin of bacterial resistance to penicillin (Demerec, 1945*a*,*b*) indicate that differences in resistance can be explained by assuming the mutation of a number of equally important genes. Unlike penicillin resistance, which increases stepwise, and is partially dependent on selection, the appearance of streptomycin resistance (Demerec, 1948) seems to result from the mutation of genes of different potencies, thereby explaining the random appearance of progeny exhibiting various degrees of resistance to streptomycin. The extreme variability of streptomycin sensitivity exhibited by multiple fishings of the present strain of *Staph. aureus* both before and after initial exposure to streptomycin, is amenable to such an explanation.

The mechanism by which these variants of *Staph. aureus* utilize streptomycin has not been established. Dihydrostreptomycin is the only substance of those so far tested which could be substituted for streptomycin. It seems evident from the experimental results that the growth-stimulating factor is a moiety of streptomycin, rather than a trace contaminant. The inability of inactivated streptomycin to support or stimulate the growth of these variants suggests utilization of a molecular structure intimately related to the inhibiting properties of streptomycin.

If the individual characteristics of a given cell are considered as an expression of enzyme structure, then a change of cell character must reflect a change in enzyme structure. The ability to utilize streptomycin, or some moiety thereof then indicates the action of a new enzyme system. The concurrent loss of ability to grow in the absence of this new substrate further indicates the injury or loss of that system which previously had been utilized by the cell in obtaining from other sources that metabolite which must now be obtained in the whole or in part from streptomycin.

It is well established that the capacity to elaborate enzymes under appropriate substrate stimulation, in yeasts at least, is gene determined and is inheritable in Mendelian patterns (Lindegren, 1945; Lindegren, Spiegelman & Lindegren, 1944). If this basis for enzyme elaboration holds for *Staph. aureus*, the logical assumption then would be that streptomycin dependence, like streptomycin and penicillin resistance (Demerec, 1945*a*,*b*, 1948), is a potentiality of a few individual cells in any large bacterial population. The spontaneous appearance of streptomycin-dependent variants in this strain of *Staph. aureus* can be explained readily on this basis, i.e. a rapidly mutating strain, a few cells of which carry a recessive gene determining the elaboration of an enzyme suitable for the utilization of streptomycin.

The significance of the pleomorphic changes reported here is not fully understood. However, the occurrence of similar morphological changes, appearing either spontaneously or in response to an altered environment in a number of different species of bacteria (Dienes, 1939–47) suggests a common cell mechanism, whether such phenomena are regarded as simple response to injury or as representing something more fundamental to the continued growth and survival of the cell. The appearance of pleomorphic change in a few cells

when large inocula of this strain were transplanted to streptomycin-free agar, concurrent with the disappearance of streptomycin dependency suggests a relationship between morphological variation and mutation.

The substance of this paper was presented in part at the first meeting of the North-east Branch, Society of American Bacteriologists, on 11 February 1949 at Cambridge, Massachusetts.

The work was supported in part by a grant from The Grant Foundation Inc.

REFERENCES

Agnew, S., Kaplan, M. & Spink, W. W. (1947). Comparative inhibitory effect of penicillin and streptomycin upon the action of staphylocoagulase. *Proc. Soc. exp. Biol., N.Y.*, **65**, 38.

Bartz, Q. R., Controulis, J., Crooks, H. M. jr. & Rebstock, M. C. (1946). Dihydrostreptomycin. *J. Amer. chem. Soc.* **68**, 2163.

Bondi, A. jr. & Dietz, C. C. (1944). Production of penicillinase by bacteria. *Proc. Soc. exp. Biol., N.Y.*, **56**, 132.

Demerec, M. (1945 *a*). Production of *Staphylococcus* strains resistant to various concentrations of penicillin. *Proc. nat. Acad. Sci., Wash.*, **31**, 16.

Demerec, M. (1945 *b*). Genetic aspects of changes in *Staphylococcus aureus* producing strains resistant to various concentrations of penicillin. *Ann. Mo. bot. Gdn*, **32**, 131.

Demerec, M. (1948). Origin of bacterial resistance to antibiotics. *J. Bact.* **56**, 63.

Denkelwater, R., Cook, M. A. & Tishler, M. (1945). Effect of cysteine on streptomycin and streptothricin. *Science*, **102**, 12.

Dienes, L. (1939 *a*). L type variant forms in cultures of various bacteria. *Proc. Soc. exp. Biol., N.Y.*, **42**, 636.

Dienes, L. (1939 *b*). A peculiar reproductive process in colon bacillus colonies. *Proc. Soc. exp. Biol., N.Y.*, **42**, 773.

Dienes, L. (1942). The significance of large bodies and development of L type of colonies in bacterial cultures. *J. Bact.* **44**, 37.

Dienes, L. (1944). L type of growth in cultures of haemolytic parainfluenza bacillus. *Proc. Soc. exp. Biol., N.Y.*, **55**, 142.

Dienes, L. (1946). Reproductive processes in *Proteus* cultures. *Proc. Soc. exp. Biol., N.Y.*, **63**, 265.

Dienes, L. (1947). Isolation of pleuropneumonia-like organisms from cultures of *H. influenzae*. *J. Bact.* **54**, 3.

Dienes, L. & Smith, W. E. (1944). The significance of pleomorphism in *Bacteroides* strain. *J. Bact.* **48**, 125.

Duggar, B. M. (1948). Aureomycin—a new antibiotic. *Ann. N.Y. Acad. Sci.* **51**, 177.

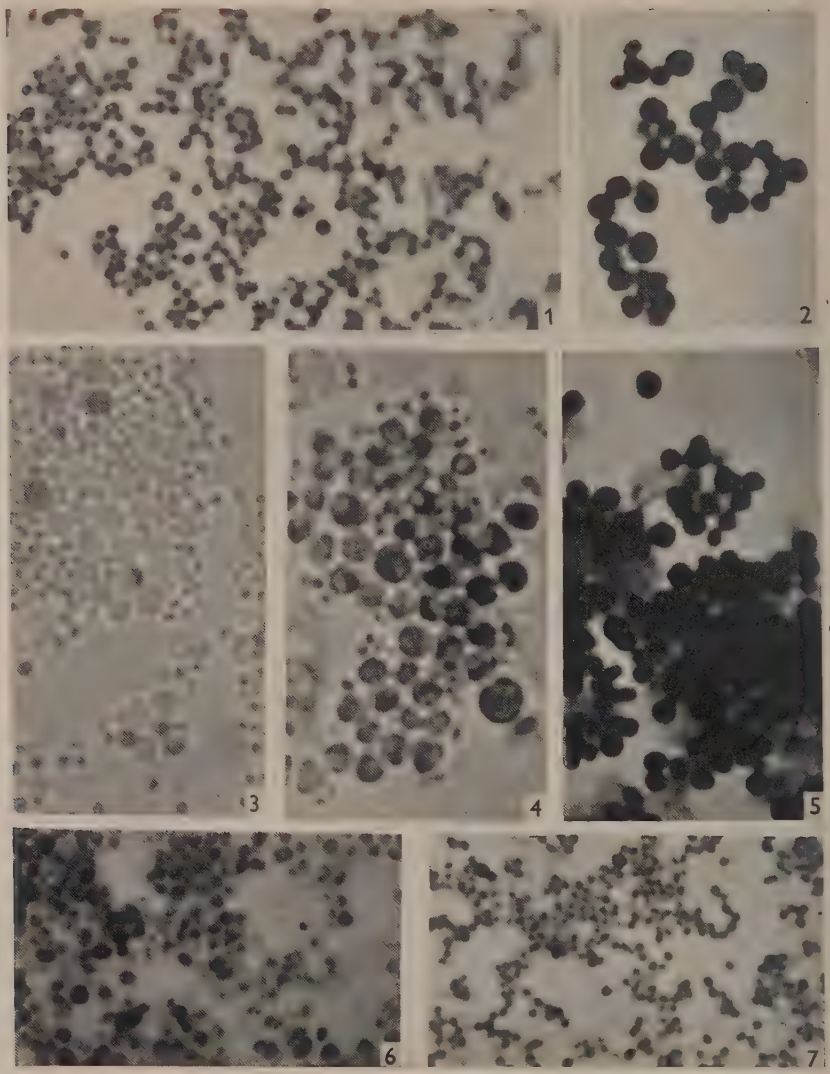
Johnson, B. A., Anker, H. & Meleney, F. L. (1945). Bacitracin; a new antibiotic produced by a member of the *B. subtilis* group. *Science*, **102**, 376.

Lindegren, C. C. (1945). Mendelian and cytoplasmic inheritance in yeasts. *Ann. Mo. bot. Gdn*, **32**, 107.

Lindegren, C. C., Spiegelman, S. & Lindegren, G. (1944). Mendelian inheritance of adaptive enzymes in yeast. *Proc. nat. Acad. Sci., Wash.*, **30**, 346.

Price, C. W., Nielsen, J. K. & Welch, H. (1946). Estimation of streptomycin in body fluids. *Science*, **103**, 56.

Rake, G. & Donovick, R. (1946). Procedure for testing sterility of concentrated streptomycin solutions. *Proc. Soc. exp. Biol., N.Y.*, **62**, 31.



Figs. 1–7

G. E. Foley & H. Shwachman—Streptomycin dependence in *Staph. aureus*. Plate 1

EXPLANATION OF PLATE

Fig. 1. Smear from unselected colony, original 24 hr. blood agar plate. Methylene blue; × 1300. Note few large, swollen cocci.

Fig. 2. Smear from 72 hr. streptomycin-free tryptic digest agar plate streaked with D variant. Gram stain; × 1500. Note size of cocci and dividing cells.

Fig. 3. Agar block preparation from 24 hr. streptomycin-free tryptic digest agar plate streaked with D variant. Methylene blue; × 1500. Note large, swollen cocci on agar among normal cocci. Notice cell wall of some of the larger cocci.

Fig. 4. Agar block preparation from 48 hr. streptomycin-free tryptic digest agar plate streaked with D variant. Methylene blue; × 1500. Normal cocci have almost completely disappeared.

Fig. 5. Impression preparation, made from same plate illustrated in fig. 4. Methylene blue; × 1500.

Fig. 6. Agar block preparation, edge of colony on plate illustrated in fig. 4. Methylene blue; × 1500. Note large, swollen cocci dividing by fission.

Fig. 7. Impression preparation from 24 hr. blood agar plate planted with colonies illustrated in fig. 3. Methylene blue; × 1300. Note reappearance of normal morphology and a few remaining large, swollen cocci, some of which are dividing.

(*Received* 11 *June* 1949)

Studies of a Yeast Exacting towards *p*-Aminobenzoic Acid

By N. S. CUTTS and C. RAINBOW
Department of Industrial Fermentation, University of Birmingham

SUMMARY: Certain single-cell strains of brewer's top fermentation yeasts require *p*-aminobenzoic acid as an essential nutrient. Relatively large quantities of adenine, together with certain amino-acids, of which methionine and histidine were of prime importance, could substitute for the *p*-aminobenzoic acid.

In the presence of adenine, histidine, and suboptimal amounts of methionine, the addition of any one of seven other amino-acids caused a marked stimulation of growth. These amino-acids were leucine, *iso*leucine, *nor*leucine, valine, *nor*valine, tyrosine and phenylalanine.

Rainbow (1948) and Cutts & Rainbow (1949) found that certain strains of *Saccharomyces cerevisiae* isolated from British brewery top fermentation yeasts required *p*-aminobenzoic acid (PABA) as an essential nutrient. The present work is a study of the nutrition of one of Rainbow's PABA-requiring strains of yeast and of its possible use in the microbiological assay of PABA.

METHODS

Two techniques were mainly used.

Auxanographic tests. For the preliminary qualitative establishment of growth requirements, Beijerinck's auxanographic test, as described by Pontecorvo (1949), was used. Plates were made of a layer of basal synthetic medium containing a heavy seeding of yeast (of the order of 500,000 cells per plate). Minute amounts of substances under test were then touched on to the surface of the agar at marked points. After incubation at 25° for 18–48 hr., the plates were examined. A zone of growth at the point of application of a test substance indicated a positive effect as a growth stimulant.

Tube tests. The information elicited by auxanographs was studied further by growth tests in tubes, by the usual procedure for microbiological assays. Tubes ($6 \times \frac{5}{8}$ in.) containing 6 ml. of test medium were sterilized for 10 min. at 10 lb. pressure. After inoculation, the tubes were incubated in a vertical position at 25° in a water bath and shaken every 24 hr. Growth was determined turbidimetrically in the Spekker absorptiometer, using a 1 cm. cell, for which 6 ml. of cell suspension is suitable. Unless otherwise stated, each response quoted in the Tables in this paper is the average Spekker reading of duplicate tubes after 72 hr. incubation at 25°.

Test organism and inocula. This work was carried out entirely with the single-cell strain of an English top fermentation brewery yeast designated 'Yeast 47' by Rainbow (1948). The organism was maintained on malt agar slants, transfers being made weekly, or more frequently when necessary. Inocula for the tube tests were prepared by transferring a little of the surface growth from a 16 to

24 hr. slope culture into sterile 0·85 % saline, shaking to a uniform suspension and inoculating each tube with one drop (about 0·03 ml.) of the suspension. Such a suspension gave a reading between 0·08 and 0·20 on the Spekker, corresponding to 20,000–50,000 cells per tube.

Medium. The composition of the basal defined medium used for all tube tests was, per 100 ml. final volume, in g.: glucose, 4·0; $(NH_4)_2HPO_4$, 0·4; KH_2PO_4, 0·2; $MgSO_4.7H_2O$, 0·1; $CaCl_2.6H_2O$, 0·025; in μg.: inositol, 1000; *d*-biotin, 0·2; Ca *d*-pantothenate, 100; aneurin, 100; pyridoxin HCl, 100; nicotinic acid, 100; riboflavin, 5; KI, 10; in ml., lactic acid (syrupy A.R.), 0·3; potassium lactate (50 %, v/v), 1·2; trace element solution (Emery, McLeod & Robinson, 1946), 0·1. Adjustment to pH 5·0–5·2 was made, if necessary, with lactic acid or sodium hydroxide.

For auxanographic tests the same basal medium was used with two modifications; the glucose concentration was decreased to 1 % and the medium was solidified with 2 % agar that had been repeatedly washed with distilled water.

RESULTS

p-*Aminobenzoic acid as a growth factor for Yeast* 47. The basal PABA-free medium supported the growth of some twenty single-cell strains of yeast isolated from British breweries, the essential growth factors for which were biotin, pantothenic acid and, in some cases, inositol. Four strains, however, failed to grow in this medium. Auxanographic tests identified the missing factor in each case as PABA. From these four strains, that designated Yeast 47 was selected for further study. In addition to PABA, this yeast required biotin and pantothenic acid; inositol was strongly stimulatory.

The response of Yeast 47 in basal medium to different concentrations of PABA was determined by tube tests. The response curve was sigmoid and a half-maximum growth was produced by *c.* 0·007 μg. PABA/ml. medium (Table 2). In the absence of PABA, Yeast 47 failed to grow appreciably.

Replacement of PABA by purines and amino-acids. Snell & Mitchell (1942) and Shive & Roberts (1946) found that certain purine bases and methionine were effective in reversing sulphanilamide inhibition whilst Lampen, Roepke & Jones (1946) found that an amino-acid medium containing adenine could support the growth of a PABA-requiring mutant of *Escherichia coli.* Tests were therefore made to see whether purine and pyrimidine bases and amino-acids could replace PABA for Yeast 47.

The auxanographic technique was used for the preliminary experiments. To test the bases a medium was used in which the ammonium phosphate of the basal medium was replaced by 0·4 g./100 ml. vitamin-free casein acid-hydrolysate (Ashe Labs. Ltd.). The bases adenine, guanine, xanthine, uracil, thymine and cytosine were tested singly and in pairs. PABA was included as a test substance to ensure at least one positive result. Results were read after 24 hr. incubation. A positive result was observed at those places on the plate only where adenine or PABA had been applied. The other bases had no effect even when incubation was prolonged to 96 hr. Tube tests showed that about

10 μg. adenine HCl/ml. was sufficient to induce a half-maximum growth in casein hydrolysate medium. Adenine possessed no growth-promoting activity in the absence of amino-acids.

Nature of the active amino-acids. A preliminary survey of the amino-acids responsible for promoting the growth of Yeast 47 in the presence of adenine in PABA-free media was made by the auxanographic test. For this purpose, 10 μg. adenine hydrochloride/ml. were incorporated into the basal medium and twenty-two amino-acids tested. Of these only methionine had any action. The auxanographs were then repeated, with adenine (10 μg./ml.) and DL-methionine (20 μg./ml.) incorporated in the basal medium, to test whether other amino-acids had supplementary effects. There was now a positive response to histidine, visible within 24 hr., after which period general growth of the plates took place.

Taking the auxanographs a stage further by including 10 μg./ml. histidine as well as adenine and methionine in the basal medium and testing the remaining amino-acids, it was found that any one of seven amino-acids had a positive effect after 18 hr. incubation. This group of amino-acids (referred to below as the leucine group) consisted of: leucine, *nor*leucine, *iso*leucine, valine, *nor*valine, tyrosine and phenylalanine. The response to tryptophan was also positive, but less strongly marked. DL-Alanine and DL-threonine had a positive effect on one occasion only.

Table 1. *The importance of adenine, methionine, histidine and leucine in PABA-free medium for the growth of Yeast 47*

Growth recorded as Spekker readings; half-maximum growth = 0·75; uninoculated medium reads 0·0. The supplements were as follows, in μg./ml. medium: A = 10 adenine HCl; M = 20 DL-methionine; M' = 100 DL-methionine; H = 10 L-histidine HCl; L = 10 L-leucine; P = 0·008 PABA.

Basal medium plus ...	*AMH*	*AML*	*AHL*	*MHL*	*AMHL*	*AM'H*	*AM'HL*	*P*
	Spekker readings							
Relative growth ...	0·05	0·02	0·01	0·01	0·39	0·34	0·33	0·51

The results of these auxanographic tests were confirmed by tube tests. Table 1 illustrates the importance of adenine, methionine, histidine and a member of the 'leucine group' in the nutrition of Yeast 47 when PABA was omitted from the medium. Omission of any one of these substances severely restricts growth, good growth being obtained when the three substances and one of the leucine group are present together. In Table 1, L-leucine is quoted as typical of the leucine group. The effect of the leucine was less evident, however, when the methionine content of the medium was increased, until, at 100 μg. DL-methionine/ml., no effect was observed.

Effect of adenine and amino-acids in diminishing the requirement of Yeast 47 for PABA. The growth-promoting effect of PABA for Yeast 47 could thus be imitated in PABA-free media by adenine in the presence of methionine, histidine, and, where the amount of methionine was small, by one of the leucine group of amino-acids. However, whereas the quantity of PABA required to

promote a half-maximum growth was of the order of 0·007 μg./ml., the corresponding quantities of adenine and the amino-acids were relatively large, of the order of 10 μg./ml., i.e. about 1000 times greater in weight. This suggests that PABA functions in an enzyme system involved in the synthesis of these substances.

Supporting evidence was sought by measuring the response of Yeast 47 to different concentrations of PABA in the basal medium and in media supplemented with adenine, methionine, histidine and other amino-acids. A decrease in the amount of PABA required to cause half-maximum growth (corresponding to a Spekker reading of 0·75) in the basal medium supplemented with a particular substance, as compared with the basal medium alone, is regarded as evidence that PABA is involved in the synthesis of that substance (Shive & Roberts, 1946). Supplements to the basal medium were added as follows: adenine hydrochloride, 10 μg./ml.; DL-methionine, 20 μg./ml.; L-histidine monohydrochloride, 10 μg./ml.; amino-acids of the leucine group, 10 μg. of the L-isomer/ml.

The results are set out in Table 2. The amount of PABA required to give half-maximum growth has been interpolated from curves drawn from the data

Table 2. *Response of Yeast 47 to PABA in media supplemented with adenine and certain amino-acids*

Growth recorded as Spekker readings; half maximum growth reads 0·75; uninoculated medium reads 0·0. The various supplements were as follows, in μg./ml. medium: *M* = 20 DL-methionine; *H* = 10 L-histidine HCl; *L* = 10 L-leucine; *C* = 10 L-cystine; *A* = 10 adenine HCl.

Basal medium plus	mμg. PABA/ml.							mμg. PABA/ml. for half-maximum growth	Percentage relative PABA-sparing effect
	0·0	1·67	3·33	5·00	6·67	8·33	10·00		
	Spekker readings								
	Experiment 1								
No additions	0·0	—	—	0·30	0·78	1·28	1·42	6·5	—
M	0·0	0·12	0·38	1·04	1·40	—	—	4·3	34
H	0·0	—	—	0·51	0·93	1·38	1·45	5·9	9
L	0·0	—	—	0·43	0·98	1·34	1·44	6·0	7
M+*H*	0·01	0·19	0·58	1·15	1·42	—	—	3·8	41
M+*L*	0·0	0·68	1·28	1·36	1·40	—	—	1·8	72
H+*L*	0·0	—	—	0·45	0·97	1·33	1·50	6·0	7
M+*H*+*L*	0·01	0·82	1·37	1·43	1·48	—	—	1·5	77
	Experiment 2								
No additions	0·01	—	—	0·17	0·43	0·85	1·17	7·9	—
A	0·01	—	—	0·59	0·90	1·13	1·33	5·8	26
M	0·02	0·09	0·29	0·67	1·23	—	—	5·2	34
A+*M*	0·02	0·29	0·82	1·18	1·36	—	—	4·3	61
A+*H*	0·01	—	—	0·55	0·83	1·04	1·30	6·2	22
M+*H*	0·02	0·15	0·40	0·78	1·25	—	—	4·8	39
	Experiment 3								
No additions	0·01	—	—	0·35	0·81	1·31	—	6·4	—
M+*H*	0·02	—	0·51	1·17	1·46	—	—	3·9	39
M+*H*+*C*	0·02	—	0·56	1·23	1·48	—	—	3·8	41

contained in the table. The relative PABA-sparing effects were calculated from the formula:

$$\text{percentage relative PABA-sparing effect} = \frac{x-y}{x} \times 100,$$

where x and y are the quantities of PABA required to give half-maximal growth in unsupplemented and supplemented media respectively.

The results may be summarized:

(*a*) Addition of methionine to the basal medium decreased the amount of PABA required to induce a given growth response. The half-maximum growth level was promoted by 6·5 mμg. PABA/ml. basal medium, the corresponding figure in the presence of methionine being 4·3 mμg., i.e. methionine exerts a relative PABA-sparing effect of 34 % (Table 2, Exp. 1).

(*b*) The relative PABA-sparing effect of histidine was relatively small, about 9 %. In the presence of methionine the effect of histidine was roughly additive (Table 2, Exp. 1).

(*c*) Leucine alone exerted a small PABA-sparing effect (7 %). In presence of methionine, however, a marked interaction occurred. From Table 2 the relative PABA-sparing effect of leucine in presence of methionine, calculated on an additive basis, is 41 %. The experimentally determined relative PABA-sparing effect was greater than this (72 %), indicating an interaction effect between methionine and leucine. Other members of the leucine group showed, qualitatively, the same effect. Tryptophan, alanine and threonine also exerted a PABA-sparing effect in presence of methionine, but to a much smaller degree.

(*d*) Adenine exerted a relative PABA-sparing action of 26 % (Table 2, Exp. 2). The sum of the single effects due to adenine and methionine is equal to their effect together (61 %); i.e. no interaction was observed.

(*e*) The following amino-acids showed no growth-promoting effect and possessed little or no PABA-sparing effect, either alone or in the presence of methionine: arginine, asparagine, aspartic acid, cystine, cysteine, glutamic acid, glycine, lysine, proline, hydroxyproline, serine. In Table 2 cystine is quoted as typical of these amino-acids.

DISCUSSION

The essential growth factors required by most brewery top fermentation yeasts are biotin, pantothenic acid and sometimes inositol, but strains exist which also require PABA. These strains, however, may not be suitable for the microbiological assay of PABA. In the case of the strain studied, the presence of adenine and certain amino-acids in test samples would interfere with assays.

Shive & Roberts (1946), as a result of sulphanilamide inhibition experiments on *Escherichia coli*, believe that PABA takes part in the synthesis of purines and methionine; our results support this view. In the absence of PABA, Yeast 47 responds to adenine and certain amino-acids, of which methionine is the most important. The fact that the amounts of adenine and methionine required to induce half-maximal growth are relatively large compared with the amount of PABA required to give a similar response suggests that PABA

functions in enzymic systems which synthesize adenine and methionine. Further support for this view is given by the PABA-sparing effect of methionine and adenine. Histidine showed a small PABA-sparing effect, which was significant but not so large as that of methionine. Hall (1947) found that histidine replaced folic acid as a growth factor for *Streptococcus lactis* R and thus has already established an indirect connexion between histidine and PABA, since the latter is part of the folic acid (pteroylglutamic acid) molecule. More evidence is required before an interpretation of the connexion between methionine and the members of the leucine group of amino-acids can be offered.

REFERENCES

Cutts, N. S. & Rainbow, C. (1949). Nutrition of a strain of brewer's yeast exacting to *p*-aminobenzoic acid. *Nature, Lond.*, **164**, 234.

Emery, W. B., McLeod, N. & Robinson, F. A. (1946). Comparative microbiological assays of members of the vitamin B complex in yeast and liver extracts. *Biochem. J.* **40**, 426.

Hall, D. A. (1947). Histidine and its relation to folic acid. *Biochem. J.* **41**, 299.

Lampen, J. O., Roepke, R. R. & Jones, M. J. (1946). The replacement of *p*-aminobenzoic acid in the growth of a mutant strain of *Escherichia coli*. *J. biol. Chem.* **164**, 789.

Pontecorvo, G. (1949). Auxanographic techniques in biochemical genetics. *J. gen. Microbiol.* **3**, 122.

Rainbow, C. (1948). *Para*-aminobenzoic acid a growth factor for certain brewer's yeasts. *Nature, Lond.*, **162**, 572.

Shive, W. & Roberts, E. C. (1946). Biochemical transformations as determined by competitive analogue-metabolite growth inhibitions. *J. biol. Chem.* **162**, 463.

Snell, E. E. & Mitchell, H. K. (1942). Some sulphanilamide antagonists as growth factors for lactic acid bacteria. *Arch. Biochem.* **1**, 93.

(*Received* 1 *July* 1949)

The Degradation of Starch by Strains of Group A Streptococci having Related Antigens

By NUALA CROWLEY
Bacteriology Department, Royal Free Hospital, London

SUMMARY: Strains of Group A streptococci produced an extracellular carbohydrase which degraded starch. The enzyme in crude undialysed culture filtrates bore a general resemblance to pancreatic diastase. Amylolytic activity was decreased in environmental conditions which also affected streptococcal proteinase, namely in cultures grown at 23°, in filtered broth, or after serial mouse passage. Amylase production was associated with hyaluronidase production in strains having the type-specific M antigen 4, or the non-specific T antigen 4, whereas proteinase-positive strains of the same group of closely related specific Types 4, 24, 26, 28, 29, 46 were usually amylase-negative; but the association between production of any two of the three enzymes was not complete. Hyaluronidase-positive Type 22 strains did not produce amylase, and with two exceptions, a Type 2 strain and a strain serologically unidentified, other representative serological types were amylase-negative, though some strains degraded starch in growing cultures.

The use of starch in fermentation studies of streptococci was suggested by Andrewes (1930) and by Sherman (1937), and used by Keogh & Simmons (1940), who compared endemic Australian strains with Griffith types, and found that starch was fermented by strains serologically classified as Types 1, 2, 4, 9, 10 and 28. Maxted (personal communication), using starch plates, found that ability to split starch, though commonly shown by Type 4 strains, was infrequent in other endemic British strains examined at the Streptococcal Reference Laboratory. While studying strains isolated during a survey of hyaluronidase production Crowley (1944) observed that ability to split starch was displayed by Type 4 strains (T antigen, non-specific) with the greatest hyaluronidase activity. Both polysaccharidase activities were absent, however, in Type 4 strains which were strongly proteinase positive. In this connexion Dr S. D. Elliott (personal communication) observed that proteinase added to cultures suppressed hyaluronidase activity, and suggested that intermittent proteinase production might explain variable behaviour by hyaluronidase-positive strains. The experiments described in this paper were made to establish the incidence of amylolytic activity in relation to the antigenic components, to investigate the coincidence of different polysaccharidases elaborated by the same strains and to study the effect of proteinase on the carbohydrase activity of the strains, with reference to the occasional anomalous behaviour of streptococcal hyaluronidase which has been reported.

MATERIAL AND METHODS

Source of the strains. Hyaluronidase-producing strains were isolated during 1941–3 in a survey of hyaluronidase activity in endemic group A streptococci and subsequently preserved in the dried state. The Types were identified by slide

agglutination and classified at that time as Type 22, or Type 4 (some showing cross-agglutination with Type 24 serum). These results were confirmed with type-agglutinating sera prepared in the Streptococcal Reference Laboratory. Representative Group A strains, serologically identified by precipitation of the type specific M antigen, were supplied by Dr V. D. Allison, Streptococcal Reference Laboratory, Colindale. Recently-isolated starch-positive strains were received from the same source. A proteinase-positive strain 'K43', used as a control, was supplied by Dr S. D. Elliott, and a hyaluronidase-positive strain, now known as 'McA4', derived from the original Griffith Type 4 strain deposited in the National Collection of Type Cultures, was supplied by Dr Douglas McClean in 1941.

Cultures. Glucose beef infusion broth was made by the method of Todd & Hewitt (1932) and modified by varying the brand of peptone, buffer and method of sterilization. Five batches of beef were used, but the media compared in any one experiment were prepared from broth infused from a single batch. Proteose peptone (DIFCO), Neopeptone (DIFCO) or Evans's peptone (Evans Medical Supplies, London) were used. Sodium β-glycerophosphate (2·5 %, w/v) was substituted for bicarbonate buffer (Todd & Hewitt, 1932). The broth was sterilized by autoclaving for 10 min. at 10 lb. pressure, or twice Seitz-filtered through E.K. pads. Soft agar was made by mixing one volume of specified broth with one volume of nutrient digest agar (McCartney, Park Hospital Laboratory, Lewisham). Nutrient agar plates, used freshly poured for the study of colonial morphology, were enriched with 2·0 % (w/v) proteose peptone, 0·2 % (w/v) glucose and 10–15 % (v/v) horse serum.

Concentration of culture filtrates. Cultures were grown for 2 days in 0·5 or 1 l. volumes of broth. Greater yields of enzyme per vol. medium were produced by growing strains for 2 days at 37° in soft agar, which was frozen at −10° for 2 or more days, and the liquid then extracted by thawing. This method, originated by Heimer (personal communication) to increase the yield of staphylococcal phage, was successfully adapted by Maxted (1948) for the extraction of streptococcal 'grouping enzyme'. After centrifugation the supernatant was sterilized by filtration and refrozen. Partial concentration was obtained by slow thaw fractionation, and collection of samples of approximately the same specific gravity; protein values were estimated by the copper sulphate method of Phillips, Van Slyke, Dole, Emerson, Hamilton & Archibald (1945). The filtrates were further concentrated by precipitation with 1·5 vol. acetone after chilling at −10°. The supernatant was removed and the precipitate dissolved in sodium β-glycerophosphate buffer 0·126 M at pH 7·0. Further concentration was obtained by repeated precipitation with acetone, or with 2·5 vol. ethanol.

Typing of streptococci. Type-specific M antigens were identified by the method of Swift, Wilson & Lancefield (1943) and T antigens by slide agglutination (Griffith, 1934) in duplicate cultures grown at 37° and 22° (Elliott, 1943).

Proteinase. The clotting time of milk-thiolacetate was estimated by the method of Elliott & Dole (1947), substituting boiled skimmed milk for milk powder. The destruction of M antigen was estimated by specific precipitation tests.

Hyaluronidase. Mucin clot prevention was tested by the method of McClean (1942), using umbilical hyaluronate or synovial fluid as substrate.

Viable counts were made by the method of Miles & Misra (1938).

Capsular material was demonstrated by the indian ink method of Butt, Bonynge & Joyce (1936).

Hydrolysis of starch. Two-fold dilutions of culture supernatants or filtrates were made in sodium β-glycerophosphate buffer (pH 7·0). Samples (0·5 ml.) were mixed with 1 ml. watery starch solution (0·2 % w/v, soluble starch, Analar) and incubated in a water bath at 37°. After 2, 4, 6 hr. and overnight incubation a colour reading was recorded using 1 % Lugol's iodine, added drop by drop from a pipette until the colour was maintained in incompletely hydrolysed mixtures. This method necessitated the use of four tubes for each dilution and was sometimes modified when the relative activities of strains became apparent. To minimize the personal error involved in reading a rapidly changing range of colours only grosser differences were recorded by the following scheme: + + + +, colourless supernatant, slight flocculant deposit, colourless after standing; + + +, colourless supernatant, flocculant deposit, red remaining in deposit; + +, mauve to pink supernatant, purple flocculant deposit; +, colourless supernatant, blue flocculant deposit; ±, coloured supernatant, blue flocculant deposit; a+ or ± at 2, 4 or 6 hr. that remained unchanged after further incubation was designated *β-amylase type activity*, but further progress to the erythro-dextrins, achroö-dextrins and final disappearance of coarse floccules was designated *α-amylase type activity* and could usually be distinguished in low dilutions after 4 hr. Concentrated filtrates hydrolysed starch very rapidly and it was necessary to take readings at 10 min. intervals.

EXPERIMENTAL

Hydrolysis of starch

Broth cultures. The results of iodine tests on overnight cultures (Neopeptone autoclaved broth) containing 0·5 % starch showed no correlation with results previously obtained with the same strains using starch plates. The pH value of overnight cultures in starch broth was found to be 5·0–6·0, the hyaluronidase production of positive strains was decreased sometimes ten-fold, and some proteinase-negative strains became positive. Supernatants of broth cultures without starch were then tested in two-fold serial dilutions against 0·2 % starch as substrate. The incidence of strains which split starch in these conditions is seen in Table 1. All strains positive on plates had some degree of activity in solution, but the plate test gave no indication of relative activity.

Culture supernatants. The substrate containing 0·2 % starch was selected after testing six strongly positive strains in a wide range of dilutions with varying concentrations of Analar starch. After 18 hr. at 37° it was found that all strains in dilutions 1/2 to 1/100 hydrolysed starch (+ + + + reading) in concentrations below 0·25 %, but 0·3 % and higher concentrations were not completely hydrolysed by the lowest dilutions. The different starch concentrations were obtained by diluting strong solutions (weighed out with 0·5 g.

differences) so that every concentration was duplicated once, and the middle and lower concentrations triplicated. The end-point was clear-cut and easy to read.

Table 1. *Degradation of starch by representative strains of endemic Group A streptococci*

M = type-specific representatives, M antigen identified by precipitin tests. T = non-specific representatives, T antigens identified by slide agglutination (growth at 37° and 23°). Classification of serological types is based on that of Lancefield & Dole (1946).

Fifty strains of Group A streptococci, identification of antigens		Number of strains degrading starch in various experimental conditions					
					Amylolytic activity of filtrates against 0·2 % watery solution		
		Total	0·5 % in broth culture	0·5 % in plates	α and β	β only	Inactive
Closely related specific types:*	M 4, 24, 28, 29 T 4, 24, 26, 28, 29, 46	22	16	11	7	4	11
Closely related specific types:	M 19, 23, 47 T 15, 17, 19, 23, 30, 47	5	1	—	—	—	5
	M 1	1	—	—	—	—	1
	M 6	3	—	—	—	—	3
	M 9	1	1	—	—	—	1
	M 14	2	1	—	—	—	2
	M 25	1	—	—	—	—	1
	T 2	1	1	1	1	—	—
	T 22	13	5	—	—	—	8
	Untypable	1	1	1	—	1	—
	Totals	50	26	13	8	5	32

* The antigenic analysis of these strains is shown in Table 11.

The appearance of coarse floccules in homogeneous solutions was the first naked-eye indication of change when the iodine test was still positive ('starch blue'). With larger volumes of enzyme + substrate mixtures, solutions showing even slight flocculation could be filtered rapidly, although the homogeneous solutions did not pass a No. 50 Whatman filter paper. This loss of viscosity was the first change detected in mixtures, and many strains which did not show any other activity were able to depolymerize starch solutions. Strains showed diversity both in rate and degree of attack, two types of activity analogous to those of α- and β-amylase being distinguished. This was investigated with cell-free filtrates.

Activity of cell-free filtrates

Table 2 demonstrates the production of an extracellular carbohydrase by a Type 4 streptococcus. The activity of this filtrate was dextrinogenic, like that of α-amylase, and was increased by partial concentration of protein in the fraction. Some activity was lost after precipitation with acetone, which appeared to affect α-amylase activity, but had no effect on β-amylase activity or the ability to decrease the viscosity of 0·2 % starch solution by qualitative test. The filtrate was also hyaluronidase-positive but proteinase-negative.

Table 2. *The amylolytic activity of a cell-free filtrate*

Iodine test: + + + + = colourless; + + + = trace of erythro-dextrins; + + = erythro-dextrins in deposit/red; ± / + = erythro-dextrins in supernatant, unchanged starch in deposit.

Strain 'H713' 37°; 2 days' incubation. Neopeptone autoclaved		Hydrolysis of 0·2 % aqueous starch solution. Time in water bath at 37° (hr.)				
		1	2	4	6	16+
Before concentration	Supernatant, 1000 ml.	—	+ + floccules	+ + + +	+ + + +	+ + + +
	Culture filtrate	—	+ + floccules	+ + + +	+ + + +	+ + + +
Fractionated by freezing and thawing	Concentrated to 200 ml.	+ + + + (30 min.)	+ + +	+ + + +	+ + + +	+ + + +
	Boiled for 30 min.	—	—	—	—	—
	Heated at 57° for 1·5 hr.	—	—	—	—	—
Strong fraction	Redissolved acetone precipitates	+ + + (45 min.)	+ + + +	+ + + +	+ + + +	+ + + +
Weak fraction (discarded)	Redissolved acetone precipitates	—	±	±	±	+

Relative activity of comparable filtrates. Crude filtrates derived from four strains grown in soft agar are compared in Table 3. In soft agar cultures some strains previously negative became proteinase positive irrespective of the brand of peptone in the medium. Of the four filtrates in Table 3, three contained active proteinase, clotting milk-thiolacetate mixture within 10 min. The strain 4226 remained proteinase-negative, and H713 was the only strain producing hyaluronidase. Strains R1823 and 1182 were strongly positive on starch plates.

Substrate specificity. Substrates of maize and potato starch (Thomas Kerfoot and Sons, Ltd., Vale of Bardsley, Lancs.) and potato starch (British Drug Houses Ltd.) were degraded by filtrates. Maize starch solutions, believed to contain a greater proportion of amylopectin, were separated into two fractions, a homogeneous supernatant giving a true amylose blue iodine reaction, and a flocculant deposit giving a blue-brown colour. Filtrates previously classed as β-amylase-positive failed to attack the amylase fraction, but destroyed the flocculant fraction, and α-amylase-positive filtrates attacked both fractions. All filtrates degraded glycogen, but streptococcal Group A polysaccharide was not attacked by homologous or heterologous filtrates.

Factors influencing the activity of cell-free filtrates.

The optimal conditions for activity were studied using several Type 4 filtrates in comparison with other types. Using comparable α-amylase-positive filtrates Type 4 strains showed almost identical behaviour; therefore only one strain is usually shown in the tables.

The effect of temperatures of 37° and 50° is shown in Table 4, with two concentrations of the substrate, and the effect of the hydrogen-ion concentration at five pH values in Table 5.

The effect of salt concentration. The effect of NaCl concentration was tested only on undialysed crude culture filtrates. NaCl concentrations greater than 1 % (w/v) decreased the rate of hydrolysis in distilled water and in buffer

Table 3. *Comparison of the amylolytic activity of four strains in partly concentrated crude filtrates of equivalent specific gravity and protein value*

Concentrates made by fluid extraction from frozen soft agar cultures 250 ml. Evans's peptone autoclaved broth + 250 ml. nutrient agar (digest, McCartney). Iodine test for degree of starch hydrolysis: + + + + = colourless; + + + = trace of erythro-dextrins in deposit/pink; + + = erythro-dextrins in deposit/red; + = erythro-dextrins in supernatant and deposit/purple.

Starch hydrolysis strains	Time in 37° water bath (min.)	Final dilutions of enzyme sample in buffer pH 7·0 — Degree of starch hydrolysis: 1/2	1/4	1/8	1/12	1/16
H713 Type 4	15	+ + +	—	—	—	—
	30	+ + +	+ + +	+ +	+ +	—
	45	+ + + +	+ + + +	+ +	+ +	+ +
	60	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
4226 Type 2	15	+ + + +	+ + + +	+ + +	—	—
	30	+ + + +	+ + + +	+ + +	+ +	+ +
	45	+ + + +	+ + + +	+ + + +	+ +	+ +
	60	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
R 1828 Type 24	15	—	—	—	—	—
	30	—	—	—	—	—
	45	—	—	—	—	—
	60	+	—	—	—	—
1182 Untypable	15	—	—	—	—	—
	30	—	—	—	—	—
	45	—	—	—	—	—
	60	+	—	—	—	—

Table 4. *The effect of temperature on the amylolytic activity of crude filtrates, using two concentrations of substrate, at* pH 7·0

Iodine test results for starch hydrolysis recorded as in Tables 2 and 3.

Culture filtrates of strains	Water bath temperatures	Starch in substrate (%)	Starch hydrolysis after various times (min.): 10	20	30	40	50	60
H713	37°	0·2	+ +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
		0·5	—	+	+ +	+ + +	+ + +	+ + +
	50°	0·2	—	—	—	—	—	—
		0·5	—	—	—	—	—	—
4226	37°	0·2	+ +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
		0·5	+ +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
	50°	0·2	+	+	+	+	+	+
		0·5	±	±	±	±	±	±

(pH 7·0). Hydrolysis was most rapid in distilled water when the NaCl concentrations were less than 0·1 %, but in buffer the greatest activity was found in two ranges of NaCl concentration, namely 0·6–0·9 % and 0·1–0·05 %. These results suggested that a high sodium-ion concentration was inhibitory,

but no conclusions have been drawn about the effect of chloride-ion concentration (Table 6).

The effect of enzyme inhibitors. McClean (1942) reported inhibition of hyaluronidase by heparin, and monoiodoacetic acid inactivates proteinase (Elliott, 1945). Sodium thiolacetate and NaCl were included as controls in the experiment shown in Table 7.

Table 5. *The effect of different hydrogen-ion concentrations on amylolytic activity*

Hydrolysis of 0·2 % starch solution after 30 min. at 37° at various pH values. Degree of hydrolysis measured by iodine test and recorded as in Table 4.

Culture filtrates of strains	pH values: 4·6	5·8	7·0	7·4	8·2
	Degree of starch hydrolysis				
H713 Type 4	+	+	++++	++	++
4226 Type 2	+	+	++++	+	+
R1823 Type 24	—	±	±/+	+	+
1182 Untypable	—	—	±	±	±

Table 6. *The influence of* Na^+ *concentration on the amylolytic activity of comparable crude filtrates*

Iodine test for degree of starch hydrolysis as in previous tables. Starch solution (0·2 %, w/v) in phosphate buffer (pH 7·0) containing NaCl as shown (%). Hydrolysis after 1 hr. at 37°.

	Strains	NaCl (%): 2·0–1·0	0·9–0·6	0·5–0·05	0·04–0·01	In water at pH 6·8
		Degree of starch hydrolysis				
Crude filtrates undialysed	H713 Type 4	±	++++	+++	++++	++++
	McA4 Type 4	+	++++	++	++++	++++
	4226 Type 2	+	++	++++	++	++++
	1182 Untypable	±	±	±	±	±

It is not clear whether the inhibitory effects of heparin and monoiodoacetic acid indicate differences in the strength of the filtrates, or a fundamental difference between the strains. In higher concentrations heparin inactivated both filtrates, but monoiodoacetic acid (0·1 M) did not completely inactivate the filtrate 4226. The retarding effect of sodium thiolacetate on one filtrate may have been due to a change of hydrogen-ion concentration (pH value 5·0–5·4) which occurred after incubation for a few minutes at 37°.

Factors influencing production of streptococcal amylase

For convenience the extracellular carbohydrase activity which has been demonstrated is now referred to in terms of the substrate, but there is no evidence of absolute substrate specificity.

Cultural conditions. Strains were grown for carbohydrase production in Neopeptone autoclaved broth with phosphate buffer, pH 7·4–7·6 (Rogers, 1944), and in proteose peptone autoclaved broth (bicarbonate buffer, pH 7·8) for proteinase production, but to study the effect of proteinase it was desirable to produce all the enzymes in the same medium. Bicarbonate buffer did not maintain a pH above 7·0 for so long a period as sodium β-glycerophosphate, therefore the first modification was a change of buffer while retaining proteose

Table 7. *The influence of heparin, monoiodoacetic acid and sodium thiolacetate on the activity of streptococcal amylase, at* pH 7·0

Degree of hydrolysis of starch measured by iodine test recorded as in Table 4.

Culture filtrates from strains	Hydrolysis time 37° water bath (min.)	Substrate starch solution (0·2 %) in phosphate buffer with addition of: Nil	NaCl 0·5 % (w/v)	Sodium thiolacetate 0·01 M	Monoiodo-acetic acid 0·001 M	Heparin* 0·01 % (w/v)
		Degree of starch hydrolysis				
H713	20	++++	++	++++	—	—
	40	++++	+++	++++	—	—
	60	++++	+++	++++	—	—
4226	20	++++	++++	++	+++	+
	40	++++	++++	++	+++	+
	60	++++	++++	++	+++	+

* Heparin: 10 ml. of 5000 unit solution in 40 ml. of buffer (Evans Sons, Lescher & Webb, Runcorn; 100,000 units = approx. 1 g.).

peptone. In an attempt to increase growth, on the assumption that this would also increase enzyme production, the broth was sterilized by filtration. In spite of profuse growth, the production of all the enzymes was diminished. The influence of cultural conditions on production was then investigated with reference to peptones and method of sterilization. The results obtained with four modifications of the same basal broth are shown in Table 8.

The number of viable organisms was greatest in proteose peptone broths. Proteinase was not found in any culture. Hyaluronidase (strain H713) was found in Neopeptone autoclaved broth in high titre, in proteose peptone autoclaved broth in lower titre, but was much decreased in both filtered broths.

The effect of incubation at 23°. Elliott (1943) found that incubation at 22° 'unmasked' T antigens of Group A streptococci which could not be detected at 37°, and that proteinase production was also suppressed at 22° (Elliott, 1945). In the previous experiment it was found that the same cultural conditions affected all three enzymes; the experiment was repeated incubating cultures at 23°. Rapidly growing cultures (37°) were inoculated into 100 ml. of broth in bottles which were incubated at 23° for 5–7 days. Samples were removed daily and kept frozen at −10° until the sets were complete; all tests were then made together using the same batches of substrate. (The same procedure was

followed in the 37° series.) The results are shown in Table 9. After 1·5 days' incubation the number of viable organisms was greater in 23° cultures.

Table 8. *The effect of brand of peptone and method of sterilization on the production of amylase*

Strain	Buffered infusion broth	Cultural conditions: Incubation 37° (days) 1	2	3
		Hydrolysis of starch		
H713 Type 4	Neopeptone autoclaved	+++	++++	+
	Neopeptone filtered	±	±	±
	Proteose autoclaved	±	++	+
	Proteose filtered	—	—	±
4226 Type 2	Neopeptone autoclaved	+++	++++	++
	Neopeptone filtered	±	±	+
	Proteose autoclaved	+	+	+
	Proteose filtered	—	±	±

++++ = α-amylase activity to achroö-dextrin.
+++ = α-amylase activity to erythro-dextrin achroö-dextrin.
++ = α-amylase activity to erythro-dextrin.
+ = β-amylase activity, observed after 2 hr.
± = β-amylase activity, slight, after 4–6 hr.

Table 9. *The effect of incubation at 23° on amylase production*

Hydrolysis of 0·2 % soluble starch by culture supernatants the final readings being made after overnight incubation at 37°.

Cultural conditions: Strain	Broth	Temperature of incubation	Period of incubation (days) 1	2	3	5	7
			Degree of hydrolysis of starch				
H713	Neopeptone autoclaved broth pH 7·4–7·6	37°	+++	++++	+	+	±
		23°	—	—	+	+	+
4226	Neopeptone autoclaved broth pH 7·4–7·6	37°	+++	++++	+	+	±
		23°	—	±	±	++	+

Symbols as in Table 8.

The depression of α-amylase activity was more striking in Neopeptone filtered broth and both proteose peptone broths. These results could not be

explained on grounds of poorer growth at a lower temperature. Hyaluronidase production was influenced in marked degree by the cultural conditions which affected α-amylase.

The effect of re-incubation at 37° *of cultures grown at* 23°. After 3 days at 23° cultures were re-incubated at 37° with the cells still present, and amylase production increased; but when the broths were filtered before re-incubation at 37° no change was found. If the broth cultures were not sterilized by one filtration, when scanty growth appeared after 2 or 3 days, amylase production was slightly increased suggesting that viable organisms must be present to initiate enzyme production at 37°. Filtrates known to be unsterile were divided into 10 ml. volumes, and 1 mm. loopfuls of various growth factors, enzyme inhibitors and possible activators added before re-incubation at 37°. The substances added can be divided into three groups: (1) *growth factors*, added on the assumption that a heat labile inhibitory factor was destroyed by autoclaving; these were aneurin, nicotinamide, nicotinic acid, riboflavin, yeast nucleic acid, liver extract ('Anahaemin', British Drug Houses Ltd.), and casein hydrolysate ('Casydrol', Glaxo Laboratories); (2) *enzymes already known to activate streptococcal proteinase*, namely 1 % papain at pH 7·0 and 1 % commercial trypsin at pH 8·0; (3) *substances or environmental alterations included on general grounds*, namely starch powder and solution, potassium hyaluronate, and an atmosphere of 10 % carbon dioxide and adjustment to pH 8·0. All substances of group (1) stimulated heavy growth overnight but without corresponding enhancement of enzyme production. 'Casydrol' suppressed amylolytic activity both in active control cultures and in hydrolysis tests. Papain, trypsin, CO_2 in gas phase and a pH value of 8·0 appeared to cause a slight increase in activity whether or not growth was increased, but the increase was within the limits of experimental error. These results suggest the possible existence of an inactive precursor of streptococcal amylase analogous to the precursor of streptococcal proteinase (Elliott & Dole, 1947); this possibility has not yet been fully investigated.

The effect of mouse passage. With the primary object of recovering M antigen in order to determine the specific types of amylase-producing strains of the T antigen groups 4, 24, 26, 28, 29 and 46, four strains were chosen for mouse passage. Two proteinase-positive polysaccharidase-variable strains, and two polysaccharidase-positive proteinase-variable strains (all agglutinated by Type 4 serum) were selected at random from the collection of old cultures. The strains were mouse avirulent and M antigen could not be demonstrated by precipitin tests. The effect of serial mouse passage on enzyme production is shown in Table 10. Capsules could not be demonstrated in any strain immediately after the last passage.

The influence of proteinase on polysaccharidase activity in the same cultures

Factors affecting proteinase production also affected polysaccharidase production, therefore the effect of proteinase alone has been difficult to assess. In the absence of proteinase, proteose peptone did not promote α-amylase production, but on the other hand proteinase was activated even in the presence

of Neopeptone when some strains were grown in soft agar. Nevertheless, there was a quantitative decrease in α-amylase production in cultures containing both proteose peptone and active proteinase. Unfortunately, the proteinase inhibitor monoiodoacetic acid had an uncertain effect on amylase activity, and a convincing controlled experiment was not made. Evidence of inhibition by active proteinase in growing cultures is circumstantial; all that can be reported at present is that Type 4 strains, which were strongly proteinase-positive

Table 10. *The suppression of polysaccharidases and proteinase after mouse passage; recovery of type specific M antigen*

Amylase + + + + = α-amylase activity. 0/± = slight β-amylase activity. Hyaluronidase + + + + = MCP titre 1/2000. 0/+ = 0–1/200, using synovial fluid; 0 using umbilical hyaluronate. Proteinase + + + + = coagulation of milk thiolacetate mixture within 15 min., in tube containing 1/4 initial dilution of sample.

Strain injected into mice		Enzyme production in 2-day cultures (37°) in favourable broth			
		Amylase	Hyaluronidase	Proteinase	M antigen
H713	Before passage	+ + + +	+ + + +	—	—
	After passage	0/±	±/0	—	4
K79	Before passage	+ + + +	+ + + +	—	—
	After passage	—	—	—	4
555/E	Before passage	—	0/+	+ + + +	—
	After passage	—	—	—	4
M135	Before passage	—	0/+	+ + + +	—
	After passage	—	—	—	4

without recourse to artificial activation, were always found to be α-amylase-negative and hyaluronidase-negative or weak-variable. The circumstantial evidence is presented in Table 11.

DISCUSSION

Using methods different from those of Keogh & Simmons (1940) it was found that though many strains attacked starch in broth cultures, extracellular amylolytic activity was confined, with two exceptions, to certain strains of one T antigen group. Moreover, the production of α-amylase by Type 4 strains was associated, before mouse passage, with hyaluronidase production in the same culture. Until recently Type 4 strains having M antigen and polysaccharidase activity at the same time had not been encountered, but fifteen Type 4 (M) strains isolated from an outbreak of streptococcal infection have now been found which possess polysaccharidase activity but show considerable strain variation both in hyaluronidase and amylase production. Familial epidemic capsulated strains (various types), which were hyaluronidase-negative, were previously found to be identical (Crowley, 1944). The present paper reports the results from the first opportunity of investigating the possibility that polysaccharidase activity increases with antigenic degradation of the strain. Though Type 4 strains have shown a greater range of polysaccharidase activity as compared with Types 24, 28 and 29 of the same group, this also depended on other biological components present in test cultures.

Table 11. *Correlation of polysaccharidases with proteinase activity in T antigen group of closely related specific Types* 4, 24, 26, 28, 29, 46

A = proteinase activated after agar passage. 0/+ = designations as in Table 10.

No. of strains showing activity	Amylase	Hyaluronidase	Proteinase	
7	++++	++++	—	A
1	+	++	—	A
3	—	++	—	
4	—	0/+	++++	
1	—	—	++++	
2	+	—	0/+	A
1	+	—	0/+	A
3*	—	—	—	A
22				

Strains specifically identified by M antigen

		Amylase	Hyaluronidase	Proteinase		
Type 4	2	++++	++++	—		Old strains
	2	—	0/+	++++		
	2	+/+++	0/++	—		New strains; two representatives showing minimum and maximum
Type 24	1	+	—	0/+	A	
Type 28	1	+	—	0/+	A	
Type 29	1	—	—	—		

* All these strains persistently mucoid after years in artificial environment.

The effect of peptones on growth of streptococci has been studied by Dole (1946), on streptococcal proteinase by Elliott (1945) and on streptococcal nucleases by McCarty (1948). Elliott & Dole (1947) also found that a dialysate broth was unfavourable for proteinase production. The findings with filtered broth as compared with heated broth suggest that a heat-labile factor delays autocatalytic processes. Growth at 23°, which is favourable for cell antigens, was also unfavourable for carbohydrase production, and the metabolism may be similar to that in filtered broth. Considering the mouse merely as a culture medium, the results of passage show a more marked though similar effect. Failure of enzyme production in the presence of new growth initiated by yeast nucleic acid and other growth factors may also be interpreted in this way. The combined results support the hypothesis that environmental conditions favouring the synthesis of cell antigens are unfavourable for the production of these enzymes.

The similarity between mucolytic and amylolytic activity was noted by Robertson, Ropes & Bauer (1940). McClean & Hale (1941) found no correlation between the hyaluronidase activity of *Clostridium septicum* and *Cl. welchii* and their ability to decrease the viscosity of starch pastes, but they noted that NaCl decreased both the amylolytic activity of *Cl. septicum* and the activity of bacterial hyaluronidases. Many streptococcal filtrates decreased the viscosity of starch solutions but failed to show further amylolytic activity. It is now

known that many Group A streptococci can alter the viscosity of three substances, hyaluronic acid, starch, and desoxyribonucleic acid as reported by McCarty (1948). In view of the opinion held by Baldwin (1948) that nucleases are pyrophosphatases it is interesting to note that Robertson, Ropes & Bauer (1940) postulated that a phosphatase catalysed the first stage in viscosity change of gastric mucin. It seems possible that depolymerization of viscous substances by Group A streptococci may be a function of one enzyme of low specificity, comparable with the relative substrate specificity shown by some phosphatases. The close similarity of the methods of attack on two large molecules by the same culture filtrate also suggests that a further stage in the degradation of starch and hyaluronate may be catalysed by one relatively specific polysaccharidase. It became apparent in the course of this study that the polysaccharidases of Type 4 and Type 22 strains decomposed the substrate differently, each type having its own specific reaction-path, perhaps determined by the antigenic structure of the strain. The use of a second polysaccharide substrate as a screening test may reveal further strain differences, and other macromolecules might be used instead of starch for amylase-negative strains. Starch has advantages as a substrate for use in multiple tests since cheap and reasonably standardized preparations are readily available.

It may be mentioned here that the primary object of this paper was not to add yet another extracellular enzyme to the battery already displayed by Group A streptococci, but to find a reason for the association of the mucin clot-preventing enzymes with certain antigenic components. Any attempt to interpret the biological significance of streptococcal hyaluronidase without reference to all the cellular components may be as delusive as the interpretation of the significance of hyaluronic acid, which, as Meyer (1947) pointed out, is a large molecule not well understood. It also seems possible that the concept of absolute substrate specificity may have hypnotized some investigators of Group A streptococcal enzymes. Polysaccharidase activity *in vitro*, whether confined to strains with related antigens in the circumstances described here, or demonstrated elsewhere by unrelated strains in different test conditions, is on the whole exceptional. As the optimal conditions for *in vitro* production seem unlikely to pertain *in vivo*, it may be reasonable to regard such strains as deviating from the usual rule. The explanation for this may possibly be found in the specific growth requirements and adaptability of the strains. If further evidence of an inactive precursor is forthcoming, the *in vivo* mechanism of hyaluronidase production might be explained. The biological significance of streptococcal hyaluronidase may have to be interpreted in relation to all the cellular components, and though the exceptional strains may yet prove the rule, it is still uncertain what that rule may be.

Part of this work was done at the Streptococcal Reference Laboratory, and I wish to thank Dr V. D. Allison for this facility. At Colindale, Mr W. R. Maxted gave me valuable suggestions and generous help with precipitin tests and mouse passage. I also wish to thank Miss J. Woodhouse and Miss D. Keys at the Royal Free Hospital, for their painstaking preparation of the various infusion broths.

REFERENCES

ANDREWES, F. W. (1930). Note on the fermentation of starch by certain haemolytic streptococci. *J. Path. Bact.* **33**, 145.

BALDWIN, E. (1948). *Dynamic Aspects of Biochemistry*, 2nd ed. Cambridge University Press.

BUTT, E. M., BONYNGE, C. W. & JOYCE, R. L. (1936). The demonstration of capsules about haemolytic streptococci with india ink or Azo blue. *J. infect. Dis.* **58**, 5.

CROWLEY, N. (1944). Hyaluronidase production by haemolytic streptococci of human origin. *J. Path. Bact.* **56**, 27.

DOLE, V. P. (1946). A dialysate medium for the culture of Group A streptococci. *Proc. Soc. exp. Biol., N.Y.*, **63**, 122.

ELLIOTT, S. D. (1943). Type relationships among Group A streptococci. *Brit. J. exp. Path.* **24**, 159.

ELLIOTT, S. D. (1945). A proteolytic enzyme produced by Group A streptococci with special reference to its effect on the type specific M antigen. *J. exp. Med.* **81**, 573.

ELLIOTT, S. D. & DOLE, V. P. (1947). An inactive precursor of streptococcal proteinase. *J. exp. Med.* **85**, 305.

GRIFFITH, F. (1934). The serological classification of streptococcus pyogenes. *J. Hyg., Camb.*, **34**, 542.

KEOGH, E. V. & SIMMONS, R. T. (1940). Cultural methods as an aid in type differentiation of Group A haemolytic streptococci. *J. Path. Bact.* **50**, 137.

LANCEFIELD, R. C. (1940). Type specific antigens, M and T, or matt and glossy variants of Group A streptococci. *J. exp. Med.* **71**, 539.

LANCEFIELD, R. C. (1940–1). Specific relationship of cell composition to biological activity of haemolytic streptococci. *Harvey Lect.* **36**, 251.

LANCEFIELD, R. C. (1943). Studies of the antigenic composition of Group A streptococci. *J. exp. Med.* **78**, 465.

LANCEFIELD, R. C. & DOLE, V. P. (1946). The properties of T antigens extracted from Group A streptococci. *J. exp. Med.* **84**, 449.

MAXTED, W. R. (1948). Preparation of streptococcal extracts for Lancefield grouping. *Lancet*, ii, 255.

MCCARTY, M. (1948). The occurrence of nucleases in culture filtrates of Group A streptococci. *J. exp. Med.* **88**, 181.

MCCLEAN, D. (1942). The *in vivo* decapsulation of streptococci by hyaluronidase. *J. Path. Bact.* **54**, 284.

MCCLEAN, D. & HALE, C. W. (1941). Studies on diffusing factors. *Biochem. J.* **35**, 159.

MEYER, K. (1947). The biological significance of hyaluronic acid and hyaluronidase. *Physiol. Rev.* **27**, 3.

MILES, A. A. & MISRA, S. S. (1938). The estimation of the bactericidal powers of the blood. *J. Hyg., Camb.*, **38**, 732.

PHILLIPS, R. A., VAN SLYKE, D. D., DOLE, V. P., EMERSON, K. jr., HAMILTON, P. B. & ARCHIBALD, R. M. (1945). Copper sulphate method for measuring specific gravities of whole blood and plasma. *Indian med. Gaz.* **80**, 275.

PIKE, R. M. (1948). Studies on streptococcal hyaluronic acid and hyaluronidase. *J. infect. Dis.* **83**, 1.

ROBERTSON, W. VAN B., ROPES, M. W. & BAUER, W. (1940). Mucinase: A bacterial enzyme which hydrolyses synovial fluid mucin and other mucins. *J. biol. Chem.* **133**, 261.

ROGERS, H. J. (1944). A simplified culture medium for the production and purification of hyaluronidase. *J. Path. Bact.* **66**, 284.

Rogers, H. J. (1948). The complexity of bacterial hyaluronidases. *Biochem. J.* **42**, 663.

Sherman, J. (1937). The streptococci. *Bact. Rev.* **1**, 3.

Swift, H., Wilson, A. & Lancefield, R. C. (1943). The typing of Group A streptococci by M precipitin reactions in capillary pipettes. *J. exp. Med.* **78**, 127.

Todd, E. W. & Hewitt, L. (1932). A new culture medium for the production of antigenic haemolysin. *J. Path. Bact.* **35**, 973.

(*Received* 11 *July* 1949)

The Effect of Growth-factor Deficiencies upon Fermentation of Glucose by Yeasts

By R. H. HOPKINS and R. J. PENNINGTON
Department of Industrial Fermentation, University of Birmingham

SUMMARY: The power of fermentation by yeast cells was decreased when the cultures were grown in media deficient in any of seven essential growth factors. A rapid increase in the rate of fermentation upon the addition of a growth factor was obtained in every case except that of inositol, where the response was much slower. The absence of a nitrogen source decreased the response to biotin, pantothenic acid or pyridoxin to a much greater extent than to aneurin or nicotinic acid. Differences were noted also in the behaviour of the two groups towards sodium azide, which can be explained on the assumption that biotin, pantothenic acid and pyridoxin affect fermentation only in a relatively indirect manner.

The decrease in the power of fermentation produced by pyridoxin deficiency was not observed until the pyridoxin content of the cells was only about one-fifth of the normal. Such yeast showed also an enhanced Pasteur effect. Pyridoxin deficiency produced no decrease in the proportion of glucose assimilated by resting cells.

The recognition of the importance of members of the vitamin B complex in the growth of yeast is of long standing. Ample data are now available concerning the requirements of a wide range of species for individual members of the group (e.g. Burkholder, McVeigh & Moyer, 1944; Emery, McLeod & Robinson, 1946), and, of course, some of these requirements form the basis of microbiological assays in common use. It is now known that if one or more of the following compounds or their analogues, biotin, pantothenic acid, pyridoxin, aneurin, nicotinic acid, inositol, or *p*-aminobenzoic acid, is absent from the growth medium, certain species show a partial or complete restriction of growth.

Two of these compounds, aneurin and nicotinic acid, are involved in the generally accepted scheme of alcoholic fermentation. The exact significance of the others is less clearly understood. Few studies have been reported of the direct effect of the lack of essential growth factors upon the power of fermentation. Observations of the effect upon CO_2 production in a medium otherwise adequate for growth (e.g. Atkin, Schultz & Frey, 1946) may be difficult to interpret, since any differences may result merely from different concentrations of yeast, due to differing growth rates. Williams, Mosher & Rohrman (1936) showed that a deficiency of pantothenic acid diminished the rate of fermentation, and a similar effect resulting from biotin deficiency has also been demonstrated and investigated in some detail (Winzler, Burk & du Vigneaud, 1944). The results presented here concern observations made with yeasts deficient in each of the seven above-mentioned essential growth factors.

METHODS

The effects of biotin and pantothenic acid deficiencies upon fermentation were studied with a strain of *Saccharomyces carlsbergensis* which required these factors for growth. A strain of *Kloeckera brevis* (No. Y915 of the U.S. Northern

Regional Research Laboratory, kindly supplied by Dr L. J. Wickerham) was used for observations with aneurin, nicotinic acid and inositol. Pyridoxin deficiency was studied in both these yeasts. A strain of *Saccharomyces cerevisiae* requiring *p*-aminobenzoic acid (Rainbow, 1948) made possible the study of this compound.

Stock cultures were grown for 24 hr. at 25° on malt extract agar enriched with 2 % of glucose. At monthly intervals a transfer was made into liquid malt extract + 2 % of glucose, and after growing at 25° for 24 hr. a fresh agar slope culture was made from this. There was no evidence of appreciable alteration in the vigour of growth of the subcultures made from the slopes during the whole period of the work.

Media and culture methods

All measurements of fermentation were carried out using washed cells which had been grown under conditions made as uniform as possible. The complete growth medium, from which appropriate deficient media were derived by omission, or decrease to suboptimal levels, of one of the growth factors, was as follows (the amounts given are per ml.): glucose, 50 mg.; vitamin-free casein hydrolysate, 1 mg.; $(NH_4)_2SO_4$, 2 mg.; KH_2PO_4, 1 mg.; NaCl, 0·9 mg.; $MgSO_4.7H_2O$, 0·125 mg.; $CaCl_2.6H_2O$, 0·185 mg.; $MnSO_4.4H_2O$, 1 μg.; $FeCl_3.6H_2O$, $ZnSO_4$, H_3BO_3, Tl_2SO_4, KI, $CuSO_4.5H_2O$, each 0·5 μg.; pyridoxin, 1 μg.; aneurin, 0·25 μg.; inositol, 25 μg.; D-pantothenic acid (Ca salt), 2·5 μg.; nicotinic acid, 2·5 μg.; DL-biotin, 0·0075 μg. Citric acid-potassium citrate buffer (0·02 M, pH 4·5) was also included.

In addition, *p*-aminobenzoic acid was included in the medium for use with *S. cerevisiae*, in the concentrations given below. Pantothenic acid referred to as a medium constituent means always the Ca salt of D-pantothenic acid. All the concentrations given are those realized after the addition of the yeast suspension as described below. Riboflavin (0·25 μg./ml.) or pteroylglutamic acid (0·02 μg./ml.), tested with *S. carlsbergensis* and *K. brevis*, had no effect upon the growth rates.

For growing cultures for the fermentation measurements, a transfer was made from the stock culture into 10 ml. of complete medium + 1 ml. of malt extract, and grown for 24 hr. at 30°. The yeast cells were washed twice with sterile 0·9 % saline containing also the same concentrations of other inorganic salts (except $(NH_4)_2SO_4$) as the growth medium; after resuspension, the concentration was adjusted turbidimetrically to exactly 20 μg. (dry wt.)/ml. Separate batches of 22·5 ml. of the appropriate medium, in tightly plugged 50 ml. conical flasks, were seeded with 2·5 ml. of this suspension and incubated at 30°, without shaking, for the time required. The cell crop was washed twice with M/15 KH_2PO_4 containing the other inorganic salts (except $(NH_4)_2SO_4$), and a suspension of 1 mg. (dry wt.)/ml. ($\pm$ 1 %, measured turbidimetrically) made in a similar medium. The manometric measurements were made on measured samples from such suspensions, which were stored at just above 0° and never used when more than 2 days old.

Measurement of fermentation

Micromanometers of the constant-pressure differential type mentioned by Dixon (1943) were used. The flasks (volume about 20 ml.) were shaken through a horizontal distance of 6 cm., 85–90 times/min., in a water bath at $30 \pm 0{\cdot}1°$. Suitable solubility corrections were made, and all gas volumes were corrected to N.T.P. All measurements of fermentation were made under commercial nitrogen, purified by bubbling through sodium pyrogallate solution; no measurable respiration occurred under these conditions.

In each run 1 mg. (dry wt.) of yeast was used and the volume of liquid in the manometer vessel (including the yeast suspension) was 2 ml. The liquid in the vessels normally contained glucose (3 %), $(NH_4)_2SO_4$ (0·25 %), KH_2PO_4 (M/30) and the other inorganic salts in the same concentrations as used in the growth medium. Any additions or variations are mentioned below. Inclusion of growth factors, other than the one in which the yeast was deficient, had no effect upon the rate of fermentation during the period of observation (a few hours) and consequently they were omitted. Apparently the cells contained sufficient reserves of the other essential metabolites derived from the growth medium. Additions from the side-bulb to the main compartment during the run were made in aqueous solution of volume never more than 0·2 ml. Other usual manometric techniques were used. Any change in weight of the yeast during the run was determined, if required, by turbidimetric measurement of the contents of the flask at the end.

Measurement of growth

It was desired also to record growth/time curves for the yeasts in the media used for growing cultures for the fermentation measurements. These were determined by separate experiments using 5 ml. quantities of media in flat-bottomed tubes, $10 \times 1{\cdot}5$ cm. internal dimensions. The depth of liquid in these tubes was approximately the same as in the flasks, and by using identical conditions of growth, rates of seeding, etc., it was found that the growth rates in the two types of vessel corresponded to within *c.* 10 %. Turbidimetric measurements were made directly on these tubes, which were selected for optical uniformity. For each medium a set of tubes was used, and the amount of growth after various times (increments usually 2 or 3 hr.) obtained by measurements upon separate tubes; successive measurements were not made upon the same tube since the shaking during the measurement stimulated growth somewhat. Each measurement was made with triplicate tubes. Turbidimetric measurements were carried out directly with a Spekker absorptiometer and expressed as dry weight of yeast by means of calibration curves for each yeast. Cells were never killed to stop growth before making measurements, since it was found that treatment with heat or antiseptics caused marked increases in the turbidity of the cultures, presumably connected with a slight shrinking and darkening observed in the microscopic appearance of the cells.

Growth-factor assays

The content of a given growth factor in the deficient and normal yeast was determined microbiologically in a few cases. 'Pyridoxin' assays were carried out with *S. carlsbergensis* as the test organism (Hopkins & Pennington, 1947); this assay estimates the total pyridoxin + pyridoxal + pyridoxamine. In the sequel the results of such assays are reported as 'pyridoxin'. To liberate the pyridoxin complex the samples were autoclaved at 120° for 5 hr. with 0·1 N-H_2SO_4. Assays of biotin and pantothenic acid were made with the same organism, with slight modifications in the technique. Suitable concentrations for the standard curves were 0–0·1 mμg. of biotin and 0–20 mμg. of pantothenic acid/ml.

RESULTS

The influence of a deficiency of any one of the seven growth factors upon the power of fermentation of the yeast in question was readily shown. Cells harvested from suitably deficient media, with one exception, gave a lower rate of CO_2 production than those of the same yeast grown in the complete medium. In preliminary studies of inositol deficiency it was observed that 18 hr. cultures of *S. carlsbergensis* in medium lacking inositol actually possessed a slightly greater power of fermentation than cultures of the same age from the complete medium, although, as expected, much less yeast was harvested from the deficient medium.

The progressive change in growth rate of *S. carlsbergensis* in various media was also measured (Fig. 1).

Response to addition of growth factor

Typical curves showing the effect of adding the appropriate growth factor upon the rate of fermentation of deficient yeasts are shown in Figs. 2 and 3. The percentage increases in the weight of yeast during the corresponding runs (90 min.) were: *B*, 10; *PA*, 5; *P*, 18 (*S. carlsbergensis*), 4 (*K. brevis*); *A*, 5; *N*, 11. Obviously the increase in the rate of CO_2 production in each case is far too large to be explained merely as a direct result of the increase in weight of the yeast; the rapidity of the response would also make this unlikely.

The magnitude of such responses varied with the age of the culture and the degree of deficiency, but were remarkably constant for cultures grown for the same time in any one medium. The conditions used for the above results were chosen from experience to allow a marked response. In cases where the yeast had an absolute requirement for the growth factor in question, e.g. *S. carlsbergensis* for pantothenic acid or *K. brevis* for inositol, it was essential to regulate the concentration of the growth factor in the culture medium so that the yeast was still appreciably growing at the time of harvesting. Otherwise the effect of the deficiency was much less reversible; the rate of fermentation, which was decreased almost to zero, was not increased, at least within a few hours, by addition of the growth factor. Such a condition of the cultures was associated with a great irregularity and variability in the shape and size of the

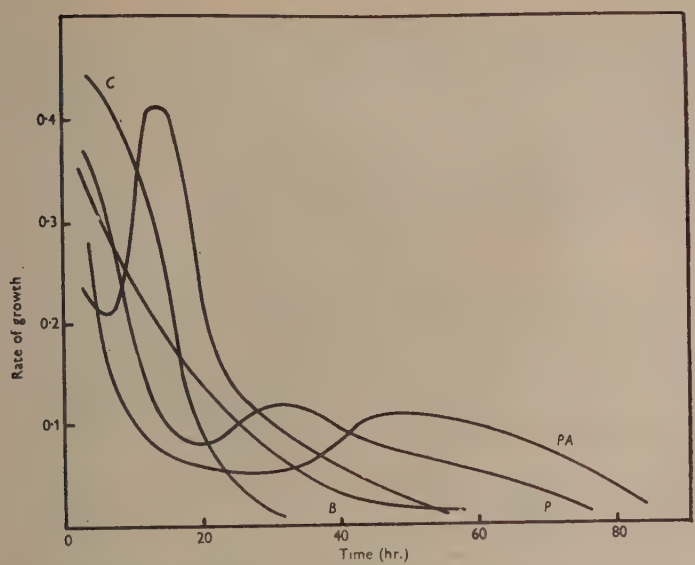


Fig. 1. Changes in growth rate of *S. carlsbergensis* with time in various media. *B*, medium without biotin; *P*, without pyridoxin; *I*, without inositol; *PA*, pantothenic acid-deficient medium; this contained 10 mμg. of pantothenic acid per ml., since growth was negligible in the complete absence of this factor. *C*, complete medium. Rate of growth $=2{\cdot}303 \times (\log_{10} Y_2 - \log_{10} Y_1)/(t_2 - t_1)$ at time $(t_1 + t_2)/2$. (Y = mg. of yeast/ml.)

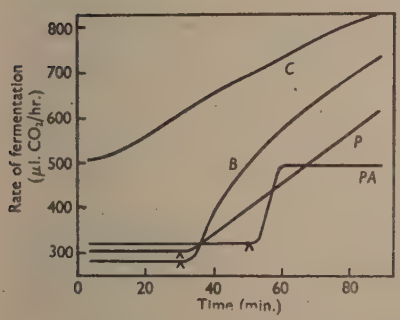


Fig. 2

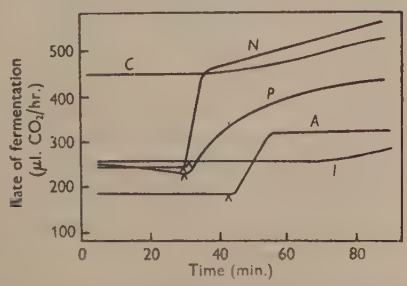


Fig. 3

Fig. 2. Response of rate of fermentation of deficient cells of *S. carlsbergensis* to addition of growth factor. *B*, biotin deficient (grown 18 hr. with no biotin); *PA*, pantothenic acid deficient (18 hr. with 40 mμg. pantothenic acid/ml.); *P*, pyridoxin deficient (18 hr. with 0·5 mμg. pyridoxin/ml.); *C*, normal (18 hr.). The following were added from the side-bulb at the times indicated: *B*, 15 mμg. biotin; *PA*, 5 μg. pantothenic acid; *P*, 2 μg. pyridoxin.

Fig. 3. Response of rate of fermentation of deficient cells of *K. brevis* to addition of growth factor. *P*, pyridoxin deficient (grown 17 hr. with 0·5 mμg. pyridoxin/ml.); *A*, aneurin deficient (16 hr. with 5 mμg. aneurin/ml.); *N*, nicotinic acid deficient (16 hr. with 20 mμg. nicotinic acid/ml.); *I*, inositol deficient (17 hr. with 1·5 μg. inositol/ml.); *C*, normal (17 hr.). The following were added at the times indicated: *P*, 2 μg. pyridoxin; *A*, 0·5 μg. aneurin; *N*, 5 μg. nicotinic acid; *I*, 50 μg. inositol.

cells, with a high proportion of elongated and filamentous forms, and many dead cells, as indicated by methylene-blue staining. Biotin, pantothenic acid and 'pyridoxin' in deficient cells of *S. carlsbergensis* were assayed. These cells contained less than 10 % of the amount of the respective growth factors found in the normal cells.

The lag in the response to inositol, evident in Fig. 3, was observed with cells of widely differing degrees of deficiency, and appears to be characteristic.

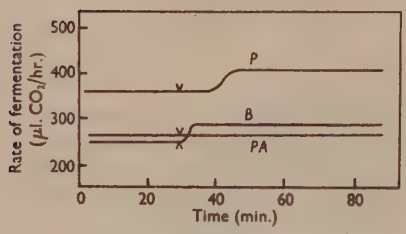


Fig. 4

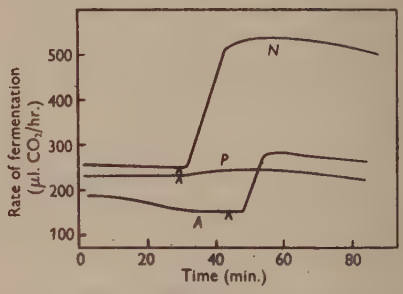


Fig. 5

Fig. 4. *S. carlsbergensis*. Response of deficient cells to addition of growth factor in absence of nitrogen source. For details see Fig. 2.

Fig. 5. *K. brevis*. Response of deficient cells to addition of growth factor in absence of nitrogen source. For details see Fig. 3.

The results obtained with *p*-aminobenzoic acid-deficient yeast, not included above, were complicated by experimental difficulties; the cells tended to adhere to the sides of the manometric vessels. Nevertheless, a definite, almost immediate response to the addition of the growth factor was clearly shown, as in the case of all the other deficiencies except inositol. The normal and deficient cultures of the yeast were grown for 16 hr. in the presence of 250 and 0·5 mμg./ml. respectively of *p*-aminobenzoic acid. (About 12 mμg./ml. were found to be necessary for maximum growth.)

The pantothenic acid-deficient cells of *S. carlsbergensis* responded equally rapidly to the addition of β-alanine; 40 μg. were added, since the potency of this substance for growth of *S. carlsbergensis* was only 1/20–1/10 (w/w) of that of pantothenic acid. It thus appears that β-alanine can be converted immediately into pantothenic acid by this yeast.

Effect of assimilable nitrogen upon the response

In order to compare further the effect of the various growth-factor deficiencies, the results of the omission of assimilable nitrogen upon the response to each growth factor (except inositol) were studied. The experiments were carried out as before except that the medium in the manometer flasks contained no $(NH_4)_2SO_4$. Typical results are shown in Figs. 4 and 5. Results with *p*-aminobenzoic acid, not recorded, indicated a greatly decreased response.

Action of sodium azide

In a further attempt to detect differences in the effect of the various growth-factor deficiencies upon fermentation, the action of sodium azide upon the rates was studied. Figs. 6 and 7 show the influence of sodium azide upon the rate of fermentation of yeasts deficient in various growth factors. In all cases several concentrations of azide other than that indicated were tested, but in no cases were the effects greater than that illustrated. The azide had no visible effect upon the microscopic appearance of the cells, and the proportion staining with methylene blue was much less than 1 %.

Figs. 8 and 9 show the effect of adding the growth factors after the addition of azide. The azide was included in the medium and the respective growth factors added at the times indicated. The final concentration of yeast was measured in all these cases; in none was there a recorded increase of more than 2 %, which probably does not exceed the experimental error.

The effect of azide upon the fermentation of the normal yeasts was also determined (Figs. 10 and 11). As before, several concentrations of sodium azide were tested in order to obtain the maximum stimulation. No stimulation could be observed at any concentration under the conditions of Fig. 10*a*; appreciably higher concentrations than that shown were inhibitory. Since yeast extract markedly increases the growth rate of *K. brevis* on the synthetic medium (in contrast with *S. carlsbergensis*, which shows only a very slight response), another test was carried out with *K. brevis* in which 2 mg. of DIFCO yeast extract were included in the fermentation medium in addition to $(NH_4)_2SO_4$. The rate of fermentation showed a progressive rise; subsequent addition of azide (added after 40 min. in two separate runs to produce concentrations of 10^{-4} M and 4×10^{-5} M) did not produce a stimulation, and in fact tended to inhibit any further rise in the rate.

Other experiments with pyridoxin

A few other aspects of pyridoxin deficiency were studied and the results are conveniently included here. *S. carlsbergensis*, grown as above, was used for all these experiments. Amongst twenty yeasts stimulated by pyridoxin, none showed relatively less growth (measured after 16 hr.) in the absence of the growth factor than this yeast.

Effect of 2:4-*dinitrophenol.* Some observations were made on the effect of 2:4-dinitrophenol (DNP) in comparison with azide. Typical results are shown in Fig. 12.

Variation in power of fermentation with concentration of growth-factor. Cultures of *S. carlsbergensis* were grown (18 hr.) with different concentrations of pyridoxin in the medium and the rate of fermentation of the washed cells measured in the usual manner. With the lower concentrations, the rate of fermentation in the manometers was constant with time; with the higher levels the rate showed a gradual increase, and in these cases the initial rate was taken. The concentration of 'pyridoxin' (= pyridoxin + pyridoxal + pyridoxamine by

S. carlsbergensis assay) in the washed cells was also determined. The results are summarized in Table 1. The yield of yeast from the growth medium and the power of fermentation of the washed cells reached a maximum at *c.* 50 mμg. of

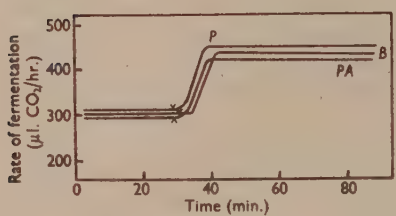


Fig. 6

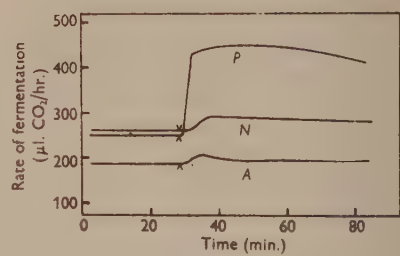


Fig. 7

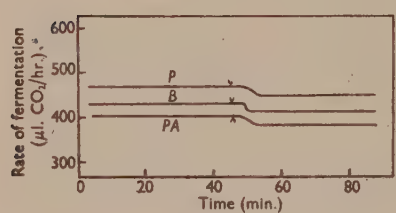


Fig. 8

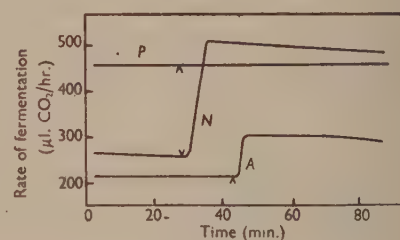


Fig. 9

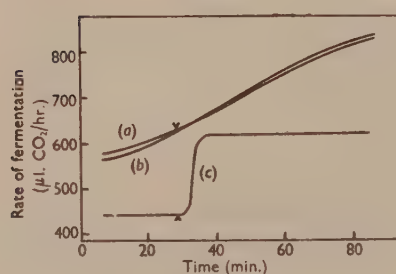


Fig. 10

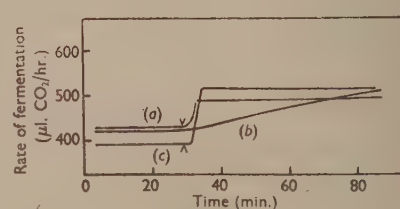


Fig. 11

Effect of sodium azide on fermentation of deficient cells.

Fig. 6. *S. carlsbergensis.* Azide added at points indicated to final concentration 10^{-5} M (*B*, *PA*); 10^{-4} M (*P*). Cells grown as for Fig. 2.

Fig. 7. *K. brevis.* Azide at 10^{-5} M throughout. Cells grown as for Fig. 3.

Fig. 8. *S. carlsbergensis.* Azide in medium initially at concentrations as for Fig. 6. Growth factors added at points shown. Cells grown as for Fig. 2.

Fig. 9. *K. brevis.* Azide in medium initially at 10^{-5} M; growth factors added at points shown. Cells grown as for Fig. 3.

Fig. 10. *S. carlsbergensis.* Cells grown in complete medium. Azide added (*a*) 2×10^{-6} M; (*b*) no azide; (*c*) 2×10^{-4} M. Fermentation medium (*c*) contained no ammonium sulphate.

Fig. 11. *K. brevis.* Cells grown in complete medium. Azide added (*a*) and (*c*) 4×10^{-5} M; (*b*) no azide. Fermentation medium (*c*) contained no ammonium sulphate.

pyridoxin/ml. medium, although the yeast then contained only a fraction of the amount of 'pyridoxin' present in the cells when grown with much higher concentrations of the growth factor. It was determined by cell counts that

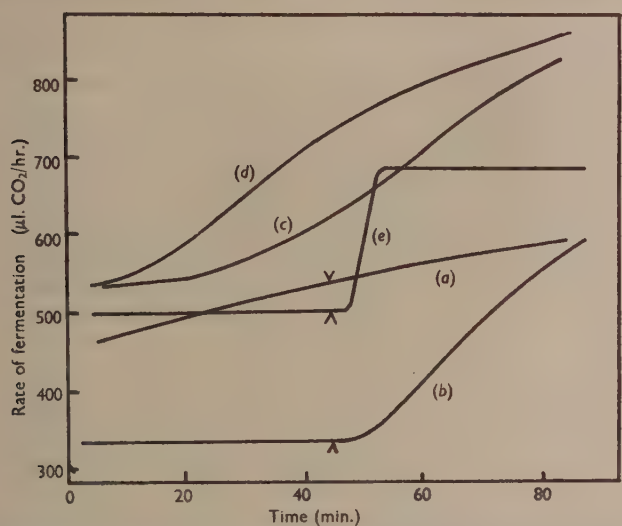


Fig. 12. Influence of 2:4-dinitrophenol (DNP) upon fermentation of pyridoxin deficient and normal cells of *S. carlsbergensis*. (*a*) and (*b*), pyridoxin deficient; (*c*), (*d*) and (*e*), normal yeast. Ammonium sulphate was omitted from the medium in (*e*). In (*a*) and (*c*), DNP (10^{-4} M and 5×10^{-5} M respectively) was present from the beginning. Pyridoxin was added in (*a*) and (*b*), and DNP (to produce a concentration of 5×10^{-5} M) added in (*e*) at the points indicated.

Table 1. *Yield of cells, 'pyridoxin' content, and power of fermentation of* Saccharomyces carlsbergensis *grown with different concentrations of pyridoxin*

Pyridoxin concentration (mμg./ml.)	Yield of yeast (mg. dry wt./ml.)	Pyridoxin content of yeast (mμg./mg. dry wt.)	Power of fermentation (percentage of control)
0	0·07	3·1	64
0·5	0·18	2·6	66
1·0	0·36	2·0	65
2·0	0·48	2·2	68
3·0	0·54	3·1	73
5·0	0·76	4·5	77
10·0	1·02	5·2	82
25·0	1·24	8·3	93
50·0	1·32	14·4	101
1000·0	1·30	67·0	100

1 mg. (dry wt.) of *S. carlsbergensis* represented *c.* 70 million cells. Thus the average amount of pyridoxin or its analogues in the yeast varied from just over 3×10^6 to *c.* 1×10^5 molecules/cell, the power of fermentation reaching its maximum at about $7{\cdot}5 \times 10^5$ molecules/cell.

Specificity of response to pyridoxin. In another series of experiments various substances commonly required as nutrients by micro-organisms were added from the side-bulb of the manometer flasks during fermentation by pyridoxin-deficient yeast; a response not shown also by normal yeast would have suggested a metabolic relationship of the substance with pyridoxin. The following substances produced no response in the rate of fermentation: a mixture of pantothenic acid (2 μg.), aneurin (0·5 μg.), nicotinic acid (2 μg.), *p*-aminobenzoic acid (2 μg.), biotin (0·01 μg.) and inositol (25 μg.); the same mixture, with the addition of pteroylglutamic acid (0·05 μg.); vitamin-free casein hydrolysate, adjusted to pH 4·5 (50 mg.) + DL-tryptophan (2 mg.); aspartic acid alone (2 mg.); 50 μg. each of adenine, guanine, uracil and xanthine; 50 μg. of choline chloride. Pyridoxal and pyridoxamine produced a response equal in rapidity and magnitude to that of pyridoxin. The same applied if the three analogues were added in limiting concentration (2 mμg.); the magnitude of the stimulation in this case was much less than when 2 μg. were added. 4-Pyridoxic acid produced no response at either concentration; this is in accordance with the negligible growth activity of this compound for *S. carlsbergensis*.

Other effects of pyridoxin deficiency. Pyridoxin-deficient yeast also showed a lowered rate of oxygen consumption in air. In this case there was a delay of over half an hour in the response to the addition of the growth factor. A similar difference in the rate of response of fermentation and respiration of biotin-deficient yeast to the addition of biotin was noted by Winzler *et al.* (1944). Aerobic CO_2 production was influenced in a like manner to anaerobic fermentation by pyridoxin deficiency; response to the growth factor was almost immediate.

The magnitude of the Pasteur effect was also measured with normal and deficient yeast. Using the numerical expression $3(Q^{N_2}_{CO_2} - Q^{O_2}_{CO_2})/Q_{O_2}$ (Meyerhof oxidation quotient; M.O.Q.), values of 8·5 and 10·2 were obtained for two cultures of pyridoxin-deficient yeast, and 4·4 and 3·4 for normal yeast. Ammonium sulphate was omitted from the manometer vessels in obtaining the results used for the computation of these values. The cultures used for measurements of the rates of respiration and M.O.Q. values were grown in shallow layers of medium, shaken continuously, instead of in the usual manner; otherwise all respiration rates were very low. It was noted incidentally that the relative yield of yeast in the pyridoxin-deficient medium was less under these conditions.

The influence of pyridoxin deficiency upon the proportion of glucose assimilated during fermentation by 'resting cells' was also investigated. It is known that many micro-organisms, even in the absence of a nitrogen source necessary for growth, do not completely oxidize or ferment added substrate, but assimilate a proportion of it (Clifton, 1946); this assimilated substrate can usually be accounted for, at least in part, by an increased carbohydrate content of the cells. (Subsequently this may be broken down at a slow rate by the organism, in the absence of further substrate.) For this experiment the glucose was tipped from the side-bulb into the yeast suspension after the manometer taps were closed; in this way the total CO_2 production from the glucose present was recorded. (Under anaerobic conditions, the cultures did not produce a

measurable amount of CO_2 in the absence of a substrate.) Only 1 or 2 mg. of pure, dry glucose were added; in other respects the medium was the same as usual, except that ammonium sulphate was omitted. When 1 mg. of glucose was added, the amount of CO_2 produced as a percentage of the theoretical by normal and deficient yeast was 77 and 72 respectively; with 2 mg., the corresponding figures were 78 and 74. It thus appears that pyridoxin deficiency does not decrease the proportion of sugar assimilated under these conditions. The weight increases corresponding to the above figures were 0·10, 0·15, 0·20 and 0·26 mg. respectively. Since the increases were due presumably to carbohydrate assimilation and not to growth in the normal sense, these figures, deduced as usual from turbidimetric measurement, are probably not correct, but may have relative significance. It was also confirmed that sodium azide inhibits such assimilation; over 90 % of the glucose was fermented in the presence of this substance (10^{-3} M).

DISCUSSION

In considering differences between the growth factors in their effects upon fermentation there is little point in comparing the magnitudes of the decreases in fermentation rates observed; these depend upon a variety of factors, such as age of culture, or whether the yeast is completely or only partly exacting for the growth factor in question. The relative power of fermentation of cells of the same species from any of the media studied was roughly in the ratio of the growth rates at the time of harvesting, deduced from the measured growth/time curves. It may be seen from Fig. 1 that the growth rate of *S. carlsbergensis* in the complete medium showed a progressive decrease with time. The absence of an appreciable lag phase was presumably because the media were inoculated with actively growing cultures. Thorne (1939) also noted a progressive decrease in growth rate of yeast cultures. Where the medium lacked pyridoxin or inositol, however, or was low in pantothenic acid (there was no measurable growth in the complete absence of pantothenic acid), an actual increase in the growth rate occurred at one period, followed by a further decrease. This was not due to a true lag phase, since the increase was preceded in each case by a fall in the rate. It can probably be interpreted as an adaptation to the deficient environment. Such an effect was not shown by *K. brevis* in any of the deficient media.

Owing to the pronounced increase in the growth rate in the inositol-deficient medium after about 10 hr., the rate after about 18 hr. was actually higher than that in the complete medium; growth rate in the complete medium had fallen appreciably owing to the already extensive growth. This difference, reflected in the metabolic activities, accounts for the relatively high power of fermentation of cultures of this age from the inositol-free medium.

The age factor should always be taken into account when studying the influence of conditions of growth upon the properties of micro-organisms. The 'physiological age', which may be different—depending upon the relative growth rates—for cultures grown for the same time under different conditions, may influence the property under investigation, thus providing an additional

variable. Apart from making observations at all stages of growth, the best procedure is probably to make comparisons between cultures with the same amount of growth.

Insufficient is known of the action of inositol to suggest a reason for the striking lag in the response seen in Fig. 3. It would seem unlikely that it forms part of a simple coenzyme functioning in alcoholic fermentation. Differences between the action of the other growth factors are suggested by a consideration of the effects of sodium azide. Sodium azide, long recognized as a respiratory poison and known to inhibit cytochrome oxidase and other Fe enzymes, has more recently been shown, by numerous workers, to inhibit growth and various other endergonic physical and chemical activities of a wide variety of organisms at concentrations lower than those at which respiration is inhibited (e.g. Clifton, 1937; Spiegelman, 1946; Winzler, 1944). On the other hand, sufficiently low concentrations may actually stimulate respiration. Fermentation by yeast may be stimulated by concentrations of azide sufficient to depress respiration, but is depressed at still higher concentrations. A clue to the nature of these effects has been provided by the observation (Spiegelman, Kamen & Dunn, 1946; Rothstein, 1946) that azide tends to prevent the disappearance of inorganic phosphate which normally occurs during the utilization of sugar by the yeast cell. The former workers made the suggestion, supported by further evidence (Spiegelman & Kamen, 1946), that azide interferes with the generation of high-energy phosphate bonds. The energy released in the breakdown processes is somehow dissipated, instead of being transferred in the usual manner by phosphorylation in the adenosine diphosphate-triphosphate system. This would explain the inhibition of endergonic processes without a depression of CO_2 production; the stimulation of CO_2 production in some cases may be interpreted (Brockmann & Stier, 1947) as being due to the release from the partial inhibition caused by the restriction of outlets for high-energy phosphate bonds.

It would thus appear that sodium azide may provide a useful means of dissociating the anabolic and catabolic phases of the cell's metabolism. It seemed possible that this effect could be utilized to reveal differences in the mechanism whereby the various growth factors influence the rate of fermentation. It is to be expected that in some cases this is merely an indirect manifestation of the action of the growth factor in processes involved in growth or other endergonic activities; that these may exercise control upon the rate of fermentation is highly probable in view of existing knowledge and ideas concerning the integration of the activities of the living cell, particularly the concept of the phosphate cycle (Lipmann, 1941). If the diminution in the rate of fermentation by a growth-factor deficiency depends upon such a mechanism, then azide, by liberating fermentation from the control of these processes, should tend to restore the rate. Likewise, a subsequent addition of the growth factor should be without effect upon the rate of fermentation. Examination of Figs. 6–9 suggests that this is the case with biotin, pantothenic acid and pyridoxin deficiencies. With aneurin or nicotinic acid deficiencies, on the other hand, the effect of azide in either respect is much less; this is in accordance with the known functions of these two factors in the reactions of fermentation.

These conclusions are substantiated by the results in Fig. 10, which fit in with the postulated mechanism of the action of azide. Fermentation by *S. carlsbergensis* is stimulated by azide only in the absence of ammonium sulphate, i.e. when growth is restricted by lack of assimilable nitrogen. The results with *K. brevis* (Fig. 11) are less clear; ammonium sulphate decreases, but does not entirely eliminate, the effect of azide. However, as stated above, the stimulation by azide in the presence of ammonium sulphate was not observed when yeast extract was also present and may therefore have been due to a deficiency of unknown factors. In confirmation of this cells of *K. brevis* grown in the defined medium with added yeast extract showed no stimulation of fermentation by azide (see Fig. 11, curve *a*).

The conclusions reached above regarding the differences in the action of the growth factors in fermentation explain also the results in Figs. 4 and 5. If the enhancement of the rate of fermentation produced by the addition of biotin, pantothenic acid or pyridoxin is, as postulated, a result of the release from restrictions imposed by reactions involved in growth, it would be expected that no response would occur where growth processes are inhibited also by the absence of available nitrogen; the responses are, in fact, greatly decreased. Ammonium sulphate produces a rapid rise in the rate of fermentation of normal cultures of *S. carlsbergensis* and *K. brevis* fermenting in the absence of a nitrogen source; this is due presumably to a stimulation of endergonic processes.

Winzler *et al.* (1944) noted that the fermentation of cells of *S. cerevisiae* deficient in biotin failed to respond to the addition of biotin in the absence of a nitrogen source, and that sodium azide prevented this response. They inclined to the conclusion that biotin and available nitrogen were involved in the synthesis of certain enzymes involved in fermentation. Such an explanation, however, is insufficient to account for the stimulation produced by azide alone.

The action of sodium azide, in dissipating the energy produced in the cell by catabolic processes, appears to be characteristic of certain other cell poisons, in particular 2:4-dinitrophenol (see review by Clifton, 1946). Early experiments with frog muscle by Ehrenfest & Ronzoni (1933) led to the conclusion that the controlling factor of lactic acid production in normal muscle is disturbed by DNP. The results in Fig 12 are similar in relevant points to those obtained with azide; the concentration of poison, however, was found to be even more critical.

Winzler *et al.* found that their biotin-deficient yeast displayed a quantitatively abnormal Pasteur effect; M.O.Q. values were several times larger than those for normal yeast (about 3–5 for the latter). The mechanisms of the Pasteur effect may be related simply to the fact that the overall rate of catabolism is limited by the prevailing concentration of inorganic phosphate or phosphate acceptors. Where the regeneration of these is slowed up by the restriction of synthetic processes, as suggested above to result from pyridoxin or biotin deficiency, it seems reasonable to expect that the Pasteur effect may be more pronounced.

REFERENCES

Atkin, L., Schultz, A. S. & Frey, C. N. (1946). *Enzymes and their Role in Wheat Technology* (ed. J. A. Anderson), Chap. xi. New York: Interscience Publishers Inc.

Brockmann, M. C. & Stier, T. J. B. (1947). Cellular mechanisms controlling rates of glucose consumption by yeast. *J. cell. comp. Physiol.* **29**, 159.

Burkholder, P. R., McVeigh, J. & Moyer, D. (1944). Studies on some growth factors of yeasts. *J. Bact.* **48**, 385.

Clifton, C. E. (1937). On the possibility of preventing assimilation in respiring cells. *Enzymologia*, **4**, 246.

Clifton, C. E. (1946). Microbial assimilations. *Advances in Enzymology*, **6**, 269.

Dixon, M. (1943). *Manometric Methods*, 2nd. ed., p. 7. Cambridge University Press.

Ehrenfest, E. & Ronzoni, E. (1933). Effect of dinitrophenol on oxidation of tissues. *Proc. Soc. exp. Biol., N.Y.*, **31**, 318.

Emery, W. B., McLeod, N. & Robinson, F. A. (1946). Comparative microbiological assays of members of the vitamin B complex in yeast and liver extracts. *Biochem. J.* **40**, 426.

Hopkins, R. H. & Pennington, R. J. (1947). The assay of the vitamin B_6 complex. *Biochem. J.* **41**, 110.

Lipmann, F. (1941). Metabolic generation and utilization of phosphate bond energy. *Advances in Enzymology*, **1**, 99.

Rainbow, C. (1948). *p*-Aminobenzoic acid a growth-factor for certain brewer's yeasts. *Nature, Lond.*, **162**, 572.

Rothstein, A. (1946). Phosphorylation in living yeast cells. *J. cell. comp. Physiol.* **28**, 221.

Spiegelman, S. (1946). The inhibition of enzyme formation. *Fed. Proc.* **5**, 99.

Spiegelman, S. & Kamen, M. D. (1946). The site of uncoupling of phosphorylation from carbohydrate metabolism in the presence of NaN_3. *Fed. Proc.* **5**, 99.

Spiegelman, S., Kamen, M. D. & Dunn, R. (1946). Mechanism of azide inhibition of synthetic activity and its relation to phosphorylation. *Fed. Proc.* **5**, 99.

Thorne, R. S. W. (1939). Mathematical representation of the course of yeast growth. *J. Inst. Brew.* **45**, 472.

Williams, R. J., Mosher, W. A. & Rohrman, E. (1936). The importance of 'pantothenic acid' in fermentation, respiration and glycogen storage. *Biochem. J.* **30**, 2036.

Winzler, R. J. (1944). Azide inhibition of anaerobic assimilation of glucose by yeast and its application to the determination of fermentable sugar. *Science*, **99**, 327.

Winzler, R. J., Burk, D. & du Vigneaud, V. (1944). Biotin in fermentation, respiration, growth and nitrogen assimilation by yeast. *Arch. Biochem.* **5**, 25.

(*Received* 14 *July* 1949)

The Isolation of *Nitrosomonas europaea* in Pure Culture

By JANE MEIKLEJOHN
Soil Microbiology Department, Rothamsted Experimental Station, Harpenden, Hertfordshire

SUMMARY: *Nitrosomonas europaea* (Winogradsky) was isolated in pure culture from Rothamsted soil. The method used entailed: (1) building up the population of nitrifiers in enrichment cultures; (2) removal of the cells from the chalk in an enriched culture with a stream of carbon dioxide; (3) picking colonies from poured silica gel plates.

The isolation of nitrifying bacteria in pure culture was first accomplished by Winogradsky (1890); it had been known for some years before that nitrification was carried out by living organisms (Schloesing & Müntz, 1877), but attempts to isolate the organisms by the usual bacteriological methods had failed (Frankland & Frankland, 1890). Winogradsky, whose brilliant insight had led him to the discovery of the autotrophic mode of life of the sulphur and iron-oxidizing bacteria, decided that the nitrifiers must also be autotrophic, and thus succeeded in isolating them by the use of media free from organic matter. At first he used his 'negative plate' method, inoculating gelatin plates and picking off from the areas where no growth could be seen. But he soon abandoned this method (1891), and obtained pure cultures by an improved technique.

He made enrichment cultures from soil on a liquid mineral medium containing an ammonium salt and a carbonate, which were renewed as they were used by the growing cultures. After a time motile bacteria appeared in the cultures, and poured plates were then made on an entirely new inorganic solid medium, silica gel. The small colonies which developed in the plates were picked off into sterile liquid mineral medium.

Warington (1891) discovered that two species were concerned in nitrification, one oxidizing ammonia to nitrite, the other oxidizing nitrite to nitrate. In 1892, Winogradsky described two bacteria, *Nitrosomonas* and *Nitrosococcus*, which oxidized ammonia, and one, *Nitrobacter*, which oxidized nitrite. In the next few years, together with Omeliansky (1899*a*), Winogradsky isolated nitrifiers from soil samples from every continent. A species from Swiss and French soils was given the name *Nitrosomonas europaea* (Winogradsky, 1904).

The reasons why nitrifying bacteria are difficult to isolate are fully described in Winogradsky's papers (1890, 1891). In the first place, nitrifiers grow very slowly, and any contaminants in enrichment cultures grow faster. It is impossible to obtain pure cultures by simple serial transfers in liquid medium; after as many as fifty transfers (Gibbs, 1919) contaminants persist, presumably living on organic matter synthesized by the nitrifiers. Secondly, nitrifiers adhere to the solid carbonate in the medium, and not all strains have a motile stage to liberate them into the liquid. Thirdly, silica gel, which is difficult to make up, is the only suitable solid medium for isolating ammonia oxidizers, and the

colonies formed on it are so small (diameter 100 μ. or less) that they cannot be seen, far less picked off, without a lens.

Winogradsky's method has been successfully used by the following authors: Boullanger & Massol (1903), Bonazzi (1919), Gibbs (1919), Nelson (1931) and Kingma Boltjes (1934, 1935). The last three workers used a micromanipulator to pick colonies, and Nelson and Kingma Boltjes used it to pick single cells. Kingma Boltjes obtained a pure culture of *N. europaea*, and his description of the species corresponds to that of Winogradsky (1904).

An entirely different method of isolation was used by Heubült (1929), Engel & Skallau (1937) and Bömeke (1939); they prepared enrichment cultures on mineral medium, made a very high dilution of the culture, put it in a shaker for an hour, and made a large number of subcultures into liquid medium; in one case sixty subcultures were made, of which five were pure. Rubentschik (1929) used gypsum blocks (Omeliansky, 1899*b*). Hanks & Weintraub (1936) spread high dilutions of enrichment cultures over the surface of silica gel plates, and picked the colonies with a micropipette. Tchan (1947) used a similar method; he sterilized the plates with ultra-violet light, and picked single cells with a micromanipulator.

A very much simplified method of growing nitrifying bacteria was described by Winogradsky & Winogradsky in 1933. They inoculated silica gel plates, 'enamelled' with a dried suspension of carbonate, with soil crumbs. When haloes appeared in the carbonate film, the growth was picked off and dilutions spread on the surface of fresh silica gel plates. This method has been criticized by Kingma Boltjes (1935), and by Hanks & Weintraub (1936), as being unlikely to give pure cultures; and indeed it is evident from a later paper by Winogradsky (1935) that it was never intended to do so. Winogradsky regarded his cultures as suitable for studies on nitrification, but not as pure in the usual bacteriological sense of the word. The method has been further criticized by Imšenecki (1946), who said that the organism described by Winogradsky & Winogradsky as *Nitrosocystis* is not really a nitrifier at all, but a myxobacterium. He claimed that the nitrification observed on plates where *Nitrosocystis* was growing was caused by *Nitrosomonas*, which developed first and was later overgrown by the myxobacteria, which used the organic matter synthesized by the true nitrifier.

EXPERIMENTAL

Method

The method used here for the pure culture isolation of *Nitrosomonas* spp. was a modification of Winogradsky's method (1891), and had three essential steps. The first was to build up an enrichment culture till it contained an adequate number of cells. The second step was the separation of the cells from the mass of carbonate at the bottom of the culture; Winogradsky (Omeliansky, 1899*a*) took advantage of the motile stage in his cultures, but my cultures did not show a motile stage, and I therefore 'blew' the cells off the carbonate with a stream of carbon dioxide. The third step was the making of poured plates of silica gel; it is impossible to streak on silica gel because it splits; and it seems

most unlikely that pure cultures can be obtained by spreading a liquid inoculum over the surface of a plate.

Enrichment culture. An enrichment culture for nitrifying bacteria was started by adding 4 g. of freshly collected surface soil from the farmyard manure plot on Broadbalk field, to 25 ml. of medium A in a 250 ml. conical flask, and incubating at 25°.

Medium A was made as follows: $(NH_4)_2SO_4$, 0·1 g.; K_2HPO_4, 0·1 g.; NaCl, 0·2 g.; $MgSO_4.7H_2O$, 0·05 g.; $FeCl_3$, trace; $CaCO_3$, 1·0 g.; tap water, 100 ml.; sterilized by autoclaving for 15 min. at 15 lb. It was modified from the classical medium of Winogradsky (Kingma Boltjes, 1935), calcium carbonate and tap water replacing the basic magnesium carbonate and distilled water of the original.

The progress of nitrification, in this culture and the transfers from it, was followed by observing the appearance and disappearance of nitrite in samples of the medium. This was done with Griess-Ilosvay reagent by a drop-plate method. Four drops of liquid, removed with a Pasteur pipette, were added to one drop of the reagent, and the colour produced compared with the colours given by a set of dilutions of a standard solution of sodium nitrite, containing 2, 5, 10, 20, 50, 75 and 100 mg./l. of nitrite nitrogen. When nitrite was absent from the sample, a rough test for nitrate was made by adding a knife-point of zinc dust to the sample and reagent (ZoBell, 1932).

Nitrification progressed rapidly in the enrichment culture; nitrite was formed at first, and then, after 14 days' incubation, no nitrite was found, but only nitrate. The quantities of nitrite formed by the transfer cultures on liquid media were much more than traces, 100 mg./l. being formed in 4–7 days, and all disappearing, with the formation of nitrate, in another 3–7 days.

To build up a population of nitrifiers in a culture it is necessary to add more ammonia at intervals as the supply is used up, and also more carbonate to keep the medium alkaline (Winogradsky, 1890). The enrichment culture was maintained for 5 months, during which time four additions of ammonium sulphate solution, totalling 0·2 g. $(NH_4)_2SO_4$, and one addition of 0·1 g. $CaCO_3$ were made to it.

Transfers on liquid media. Several replicate subcultures were made on medium A from the enrichment culture; but after two transfers on this medium some of the replicates failed to nitrify, and it was therefore decided to modify the medium. A more dilute medium, made up in glass-distilled water with a trace element mixture, was tried for two more transfers, but again some of the replicate cultures failed. It was thought that pyrophosphate in the dipotassium phosphate used might be causing the failure, and so the solid dipotassium phosphate was replaced by a solution of potassium dihydrogen phosphate, previously boiled, cooled and made up to volume. The resulting medium B was satisfactory.

Medium B was made up as follows: NaCl, 0·3 g.; $MgSO_4.7H_2O$, 0·14 g.; $FeSO_4.7H_2O$, 0·03 g.; $(NH_4)_2SO_4$, 0·66 g.; dissolved in 90 ml. glass-distilled water. KH_2PO_4 (0·1 M), 10 ml. (previously boiled); 'A–Z' trace element mixture, 1 ml., total volume, 101 ml.

Of this solution 10 ml. were diluted to 100 ml. for use, and 1·0 g. calcium carbonate added. The medium was distributed in shallow layers in conical flasks (20 ml. lots in 250 ml. flasks) and sterilized by autoclaving. *The 'A–Z' trace element mixture* (Hoagland & Snyder, 1933) contains: $LiSO_4$, 0·01 g.; $CuSO_4$, 0·02 g.; $ZnSO_4$, 0·02 g.; H_3BO_4, 0·22 g.; $Al_2(SO_4)$, 0·02 g.; $SnCl_2$, 0·01 g.; $MnCl_2$, 0·14 g.; $NiCl_2$, 0·02 g.; $CoSO_4$, 0·02 g.; $TiCl_4$ (15 % solution), 0·13 ml.; KI, 0·01 g.; KBr, 0·01 g.; distilled water, 360 ml.

The medium as used had the approximate composition: $(NH_4)_2SO_4$, 0·005 M; NaCl, 0·005 M; KH_2PO_4, 0·001 M; $MgSO_4$, 0·001 M; $FeSO_4$, 0·0001 M.

Medium B has now been in use for 2 years and twenty-four successive transfers have been made on it without any loss of nitrifying activity. The twenty-fourth transfer formed 100 mg. nitrite nitrogen/l. in 7 days, and converted it all to nitrate in 5 days more. A culture of the twenty-fourth transfer was plated on nutrient (peptone meat extract) agar and found to contain at least five contaminating species of bacteria and one actinomycete. As pointed out by Omeliansky (1899*a*) and by Gibbs (1919), it is impossible to obtain nitrifiers in pure culture by simple serial transfers in liquid medium.

Preparation of material for isolation. As a first step in the isolation of *Nitrosomonas*, the population of nitrifiers in a subculture of the twenty-fifth transfer was built up by successive additions of ammonium sulphate and calcium carbonate over a period of 4 months. At the end of this time, microscopic examination of the culture showed a large number of bacteria adhering to the calcium carbonate particles at the bottom of the flask. The next step was the removal of the bacteria from the chalk debris with a stream of carbon dioxide. A 1 ml. sample of liquid culture was added to 9 ml. saline (0·75 % NaCl) in a sterile test-tube, through the cork of which was a narrow glass tube reaching to the bottom, with its end drawn out and coiled horizontally. Through this distributing tube a rapid stream of bubbles of carbon dioxide, generated in a Kipp's apparatus and washed through distilled water, was passed for 30 min. The contents of the tube were allowed to settle, and 1 ml. samples of the supernatant carefully removed with sterile pipettes and used to inoculate poured plates of silica gel.

Plating on silica gel. A silica gel suitable for poured plates was prepared by dialysing a mixture of sodium silicate and hydrochloric acid for 3 days against distilled water, sterilizing the resulting silica sol by filtration, and setting the gel by adding a solution of nutrient salts (Kingma Boltjes, 1935).

Concentrated sodium silicate solution (10 ml.; British Drug Houses Ltd.) was mixed by gentle heating with about 20 ml. distilled water, and the volume made up to 100 ml. Concentrated hydrochloric acid (54 ml.) and distilled water (46 ml.) were mixed in a large beaker. Both solutions were cooled, and the silicate solution then poured gradually into the acid and well mixed. The mixture was poured into cellophan bags, which were suspended in cylinders full of distilled water for 3 days. The water in the cylinders was changed six or seven times, and at the end of 3 days it gave no reaction, or only a very faint clouding, with silver nitrate. During the dialysis the silica sol took up water, and the final volume was about 250 ml. The sol was sterilized by filtration

through a Ford SB pad; it must be used at once after filtration, as it gels spontaneously in about 90 min.

To make a poured plate, the following were added in succession to a sterile Petri dish: (1) the inoculum; (2) 1 ml. of the following solution, sterilized by autoclaving: NaCl, 2·0 g.; $MgSO_4$, 0·5 g.; Na_2HPO_4, 12·0 g.; KH_2PO_4, 6·0 g.; $FeSO_4$, 0·01 g.; $MnSO_4$, 0·01 g.; $NaHCO_3$, 1·0 g.; tap water, 100 ml.; (3) 1 ml. of a 5 % solution of ammonium sulphate, also autoclaved; (4) 10 ml. filtered silica sol. The plate was rotated to mix the contents, which set to a clear gel in about 10 min. at room temperature.

The isolation of ammonia-oxidizing bacteria

Silica gel plates were inoculated with the carbon dioxide-treated supernatant, and also with a ten-fold dilution of it in saline. Other plates were inoculated with dilutions (1/10,000, 1/100,000 and 1/1,000,000) made from the culture without carbon dioxide treatment. The plates were incubated at 25° for a fortnight. They were then found to be covered with numerous tiny colonies, of a peculiar glassy compact appearance, and about 100 μ. in diameter. Some of the plates were contaminated by small colonies of an actinomycete, probably a *Micromonospora*. The position of a few well-shaped and well-separated colonies was marked with indian ink under the low power of a microscope. The colonies were picked by hand from below, with a Pasteur pipette freshly drawn out in the flame to a fine point for each picking, and each colony was placed in 5 ml. of medium B in a 50 ml. flask. After incubation at 25° for 14 to 21 days, five of the flasks contained nitrite in quantity.

One of these five flasks was contaminated with an actinomycete, and was discarded. The other four, which contained only bacteria, were all from the carbon dioxide-treated material. As a test for purity, a loopful was streaked on nutrient agar. Two of the cultures, from the undiluted supernatant, gave no growth on nutrient agar in 3 weeks, and were therefore probably pure. They were plated again on silica gel, in dilutions of 1/100 and 1/10,000. The 1/100 dilution gave innumerable very tiny colonies, of about 20 μ. diameter. The higher dilution gave fewer and better developed colonies, all of the same kind (see Pl. 1). Subcultures into medium B produced 100 mg. nitrite nitrogen/l. in 6 days, and there was no further oxidation to nitrate. It may therefore be assumed that these two cultures were pure cultures of an ammonia-oxidizing bacterium.

Description of organism isolated

The bacteria in the two cultures were exactly alike. They were small oval cells, usually single, but occasionally two to four were joined end to end. Measurements on a wet preparation stained with acetic aniline blue gave the length of the cells as 1·2–1·7 μ., and the breadth as 1·0–1·2 μ. They were non-motile, and so far the motile stage has not been seen. They were difficult to stain by Gram's method because the counterstaining with safranin did not take. Eventually some good preparations were made with carbol erythrosin as a counterstain, which showed the organism to be Gram-negative. There was very

little alteration in the appearance of a growing liquid culture; the supernatant remained clear, and only after some weeks of incubation did the chalk at the bottom look slightly agglutinated. It could be seen under the microscope that the chalk particles had little masses of bacteria adhering to them. The colonies on silica gel were very small; twenty well-developed colonies measured from 80 to 140 μ. in diameter, with an average of 100 μ. They were compact and glassily refringent, colourless at first and later becoming brown. They were sometimes round or oval, and sometimes had a curious 'starfish' shape, with projecting points at different levels (see Pl. 1). Except for the lack of motility, these characters correspond to the descriptions of *Nitrosomonas europaea* given by Winogradsky (1892) and Kingma Boltjes (1935).

I should like to thank Dr Kingma Boltjes for many helpful suggestions, and particularly for showing me colonies of *Nitrosomonas* spp. I would also like to thank Mr F. A. Skinner for suggesting the use of carbon dioxide, Dr H. Lees for pointing out the toxicity of pyrophosphate, and Dr H. G. Thornton, F.R.S., for his advice and encouragement. My thanks are also due to Mr Victor Stansfield for the photograph, to Miss Mabel Dunkley for preparing the typescript, and to Miss Enid Wilsher, Miss Vera Stock and Miss Marion Chappell for technical assistance.

REFERENCES

Bömeke, H. (1939). Beiträge zur Physiologie nitrifizierender Bakterien. *Arch. Mikrobiol.* **10**, 385.

Bonazzi, A. (1919). On nitrification: III. The isolation and description of the nitrite ferment. *Bot. Gaz.* **68**, 194.

Boullanger, E. & Massol, L. (1903). Études sur les Microbes Nitrificateurs. *Ann. Inst. Pasteur*, **17**, 492.

Engel, H. & Skallau, W. (1937). Die Reincultur nitrifizierender Bakterien. *Zbl. Bakt.* (2. Abt.), **97**, 305.

Frankland, P. F. & Frankland, G. (1890). The nitrifying process and its specific ferment. *Philos. Trans.* B, **181**, 107.

Gibbs, W. M. (1919). The isolation and study of nitrifying bacteria. *Soil Sci.* **8**, 427.

Hanks, J. H. & Weintraub, R. L. (1936). The pure culture isolation of ammonia-oxidising bacteria. *J. Bact.* **32**, 653.

Heubült, J. (1929). Untersuchungen über Nitritbakterien. *Planta*, **8**, 398.

Hoagland, D. R. & Snyder, W. C. (1933). Nutrition of strawberry plants under controlled conditions. *Proc. Amer. Soc. hort. Sci.* **30**, 288.

Imšenecki, A. A. (1946). Symbiosis between myxobacteria and nitrifying bacteria. *Nature, Lond.*, **157**, 877.

Kingma Boltjes, T. Y. (1934). Onderzoekingen over nitrificeerende Bacterien. Proefschrift, Delft.

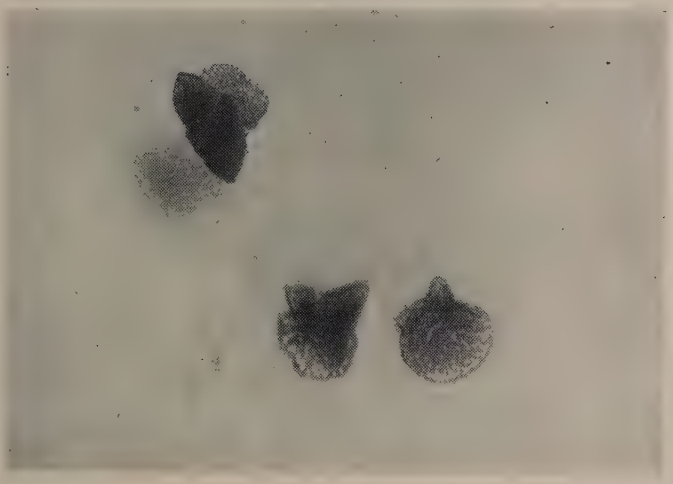
Kingma Boltjes, T. Y. (1935). Untersuchungen über die nitrifizierenden Bakterien. *Arch. Mikrobiol.* **6**, 79.

Nelson, D. H. (1931). Isolation and characterisation of *Nitrosomonas* and *Nitrobacter*. *Zbl. Bakt.* (2. Abt.), **83**, 280.

Omeliansky, V. (1899*a*). Über die Isolierung der Nitrifikationsmikroben aus dem Erdboden. *Zbl. Bakt.* (2. Abt.), **5**, 537.

Omeliansky, V. (1899*b*). Magnesia-Gipsplatten als neues festes Substrat für die Kultur der Nitrifikations-organismen. *Zbl. Bakt.* (2. Abt.), **5**, 652.

Rubentschik, L. (1929). Zur Nitrifikation bei hohen Salzkonzentration. *Zbl. Bakt.* (2. Abt.), **77**, 1.



J. Meiklejohn—Pure culture of *N. europaea*. Plate 1

SCHLOESING, T. & MÜNTZ, A. (1877). Sur la nitrification par les ferments organisés. *C.R. Acad. Sci., Paris*, **84**, 301.

TCHAN, Y. T. (1947). Contribution à l'Étude de l'Influence de l'Hyposulfite de Sodium sur quelques groupes de Microorganismes utiles du Sol. *Ann. Inst. Pasteur*, **73**, 242.

WARINGTON, R. (1891). On nitrification. (Part IV.) *J. Chem. Soc.* **59**, 484.

WINOGRADSKY, S. (1890). Recherches sur les Organismes des la Nitrification, (Parts 1–3.) *Ann. Inst. Pasteur*, **4**, 213, 257, 760.

WINOGRADSKY, S. (1891). Recherches sur les Organismes de la Nitrification. (Parts 4–5.) *Ann. Inst. Pasteur*, **5**, 92, 577.

WINOGRADSKY, S. (1892). Contribution à la Morphologie des Organismes de la Nitrification. *Arch. Sci. biol.* (St Petersburg), **1**. (Abstract in *Ann. Inst. Pasteur*, **6**, 459.)

WINOGRADSKY, S. (1904). Die Nitrifikation. In LAFAR, F. (1904–6). *Handbuch der Technischen Mykologie*, Vol. III. Jena: Gustav Fischer.

WINOGRADSKY, S. (1935). The method in soil microbiology as illustrated by studies on *Azotobacter* and the nitrifying organisms. *Soil Sci.* **40**, 59.

WINOGRADSKY, S. & WINOGRADSKY, H. (1933). Études sur la Microbiologie du Sol: (7) Nouvelles Recherches sur les organismes de la Nitrification. *Ann. Inst. Pasteur*, **50**, 350.

ZOBELL, C. E. (1932). Factors influencing the reduction of nitrates and nitrites by bacteria in semi-solid media. *J. Bact.* **24**, 273.

EXPLANATION OF PLATE

Three colonies of *Nitrosomonas europaea* on silica gel, showing 'starfish' shape. (× 120.)

(*Received* 14 *July* 1949)

Acetobacter acidum-mucosum Tosic & Walker, n.sp., an Organism Forming a Starch-like Polysaccharide

By J. TOSIC and T. K. WALKER
College of Technology, University of Manchester

SUMMARY: A new species of *Acetobacter*, *A. acidum-mucosum*, isolated from a sample of brewery yeast is proposed. In a malt extract medium at pH 4·5 or lower it forms large capsules and a voluminous viscid sediment, whereas at pH values above 4·9 the capsules are smaller and the deposit is powdery. Its ability to form a starch-like polysaccharide is a characteristic which has not been noted in the case of any other species of *Acetobacter*.

During studies of *Acetobacter* species obtained from natural sources, Walker & Tosic (1942) isolated an organism which possessed all the generic characters of *Acetobacter*, but which showed marked specific differences from eighteen authentic cultures of known species of this genus. This paper describes the characters of the new organism.

METHODS

The standardized procedure for characterization of *Acetobacter* species, described in an earlier communication (Tosic & Walker, 1946) was employed throughout. The giant colonies shown in Pl. 1, fig. 3 were prepared by Dr Dora Kulka, by the method described by Kulka, Preston & Walker (1949).

DESCRIPTION OF THE ORGANISM

Morphological characters

Shape, size and arrangement of cells. In standard malt extract at 30°, 24–48 hr. after inoculation the majority of the cells were coccoid forms while others appeared as short rods. Their dimensions varied from $0{\cdot}6 \times 0{\cdot}8\,\mu$. to $0{\cdot}8 \times 0{\cdot}8\,\mu$. In hanging-drop preparations mainly single cells were seen, but some pairs, short chains and irregular groupings were also noted. Cells taken from a malt extract agar culture after incubation at 30° for 48 hr. were mostly $0{\cdot}8 \times 1{\cdot}1\,\mu$. in size (see Pl. 1, fig. 1). *Motility* was not observed.

Thread and swollen cell formations. Cultures grown for 24 hr. in malt extract medium at 30° were raised in temperature to 40°. Sixteen hours later a large number of cells appeared as long threads and after a further 36 hr. swollen forms were also seen.

Staining. Carbol fuchsin and Loeffler's methylene blue gave satisfactory results. The Gram stain was negative. Neither *endospores* nor *flagella* were detected.

Capsules were detected by Baker's technique (Baker, 1920). The size of capsules in malt extract medium varied, depending on the pH to which the medium was adjusted. Thus, at pH 4·5 or below, cells approximately $0{\cdot}7 \times 1{\cdot}4\,\mu$.

were seen within capsules of average dimensions $3{\cdot}2 \times 4{\cdot}8\,\mu$., but some capsules were very large, even up to $8\,\mu$. in length. At pH 4·9 or above, the majority of the capsules measured $1{\cdot}6 \times 2{\cdot}0\,\mu$. and enclosed cells of dimensions $0{\cdot}6 \times 0{\cdot}8\,\mu$.

Cultural characters

Single colonies on malt extract agar plates at 25°. At 7 days, surface colonies were round, 1·5–2·5 mm. in diameter, convex with entire margin, glistening, smooth and wet in appearance, opaque and of greyish yellow colour, pasty, and the cells could readily be suspended in water. At 14 days the appearance was similar, but the average size was about 3 mm. in diameter.

Giant colony. Convex, semi-transparent, very shiny (see Pl. 1, fig. 3). For comparison there are shown on the same plate giant colonies of (I) *A. suboxydans*, a very delicate, opaque film, not coherent; (II) *A. acetigenum*, a dome-shaped, completely opaque and very coherent colony; (III) *A. acetosum*, raised growth (but not dome-shaped), opaque and of butyrous consistency.

Streak on malt extract agar at 30°. Growth visible at 24 hr. At 3 days, growth moderately strong, echinulate (lower part) and nodose, glistening, translucent with a greyish tinge. At 7 days, similar growth but becoming slightly raised and opaque. *Stab in malt extract agar at* 30°. Slight beaded growth along line of inoculation.

Streak on malt extract gelatin at 20°. Growth visible after 48 hr.; at 3 days, slight nodose growth; after 7 days, moderately strong, echinulate, greyish and glistening. Growth very weak in a stab culture.

Streak on nutrient broth agar at 30°. Growth visible after 28 hr. At 3 days, growth slight, beaded and greyish in colour. In the stab culture only slight beaded growth developed.

Streak on glucose yeast-water agar at 30°. Growth moderately strong and in appearance similar to that on nutrient broth-agar.

Growth in standard malt extract liquid medium, 30°, pH 5. Growth visible at 18 hr. At 3 days a delicate bluish ascending film had formed on the moderately turbid liquid. At 7 days turbidity was somewhat more pronounced and a scanty powdery sediment had formed. The liquid became less acid, the pH value rising from 5·0 to 6·0.

Growth in pasteurized beer. This type of growth was entirely different from that in malt extract. At 3 days a gelatinous, friable and greyish coloured surface cover had formed on the turbid medium. Turbidity increased and at 7 days a voluminous and viscid sediment had been deposited. The liquid was not ropy. In cultures in beer a strong odour of acetic acid and a fall of pH value were noted soon after growth became evident.

Nutrient broth. Growth was slower and weaker than in malt extract or in pasteurized beer. At 7 days a bluish ascending film was noted, the liquid was turbid and a scanty powdery sediment was present.

Yeast-water (10 %, w/v). Growth slight, enhanced by addition of any one of the following: ethanol, propanol, ethyleneglycol, glycerol, L-arabinose, D-xylose, D-glucose, D-fructose, maltose. Addition of lactose or of sucrose did

not stimulate growth. In all these cases an even turbidity, a delicate bluish ascending film and a powdery sediment were produced.

Effect of pH *value on the type of growth.* This was investigated on observing the difference in type of growth in such related media as malt extract and pasteurized beer. As it was shown that neither the yeast extract and hop antiseptic present in pasteurized beer, nor the lower specific gravity of the beer as compared with malt extract medium affected the type of growth, the effect of pH value was studied. It was found that malt extract adjusted to pH 4·5 or below by addition of acetic, lactic or sulphuric acid, produced the type of growth characteristic of the beer cultures. Alternatively, the same result was attained by addition of sufficient ethanol to the medium to provide, on oxidation, that quantity of acetic acid necessary to lower the pH value to below 4·5. The authors have begun a study of the viscid product formed at pH values below 4·5.

Physiological characters

Relation to temperature. In the malt extract medium the optimum range for growth was 25–30°. Slow growth occurred at 14° and in course of time the development became as strong as that at 30°. Growth at 37° was weaker than at 30° and the organism died when the inoculated medium was incubated at 42°. *Relation to oxygen.* The organism is aerobic, but tolerates a deficiency of oxygen to a greater degree than can *A. suboxydans*; but it cannot grow at an oxygen deficiency which was tolerated in a comparative study by *A. turbidans*. *Relation to hydrogen ion concentration.* In malt extract, growth is strong between pH 6·0 and 3·7. Growth was delayed at pH 7·0 and 3·10; no development at pH 3·0. *Resistance to ethanol.* Addition of 8 ml. of ethanol to 100 ml. of the malt extract delayed growth for 48 hr., and 10 ml. of ethanol increased the lag-phase of growth to 21 days.

Biochemical characters

(*a*) *Gas formation at* 30°. The organism did not produce gas in Durham tubes in any of the liquid media mentioned in this communication. (*b*) *Acid formation in different media at* 30°. Acid was produced in yeast-water cultures containing 2 % (w/v) of ethanol, propanol, ethyleneglycol, D-xylose or D-glucose. No acid was formed when glycerol, L-arabinose, D-fructose, D-galactose, sucrose, lactose, maltose, mannitol, salicin and dextrin, respectively, in yeast-water, were employed as the medium. Acetic acid was formed on oxidation of ethanol. (*c*) *Utilization of acetic acid.* In yeast-water cultures containing 2 % (w/v) of acetic acid, the organism utilized 38 % of the acid in 14 days at 30°. (*d*) *Catalase reaction*, positive. (*e*) *Voges-Proskauer reaction*, negative. (*f*) *Gelatin liquefaction*, none. (*g*) *Nutritional nitrogen requirement.* The organism cannot utilize ammonium salts as the sole source of nitrogen.

Formation of a substance giving positive tests for starch. It was noticed that the abundant surface growth produced by the organism on malt extract agar and also the cell material of giant colonies on the same medium, gave an intense blue-black coloration when treated with Lugol's iodine-potassium iodide solu-

tion. When surface growth or giant colonies of this bacterium on malt extract-agar were treated first with a solution of malt diastase (prepared from green malt) subsequent treatment with Lugol's solution failed to give the starch reaction. Controls which were treated with distilled water in place of diastase solution showed subsequently an impaired but still positive starch reaction. This suggested that the material stained was starch. None of eighteen authentic cultures of *Acetobacter* species showed this starch reaction.

CLASSIFICATION

The ability of the described organism to oxidize ethanol to acetic acid as the main product, its preference for culture media containing plant extracts (e.g. malt extract) or products derived from the latter by fermentation (e.g. beer) and the fact that it is aerobic, Gram-negative, non-spore-forming, catalase-positive and capsule-forming, clearly show that the organism belongs to the genus *Acetobacter* Beijerinck which, according to Bergey (Breed, Murray & Hitchens, 1948), is classified as Genus IV of Tribe I (Pseudomonadeae Kluyver & van Niel) of the Family Pseudomonadaceae Winslow *et al.*, in the order of Eubacteriales (Buchanan). As to its specific characters, this species has been examined under conditions of standardized technique (Tosic & Walker, 1946) in a comparative study with eighteen *Acetobacter* species: *A. aceti* (Kützing) Beijerinck, *A. acetigenum* (Henneberg) Bergey *et al.*, *A. acetosum* (Henneberg) Bergey *et al.*, *A. ascendens* (Henneberg) Bergey *et al.*, *A. capsulatum* Shimwell, *A. gluconicum* Hermann, *A. kützingianum* (Hansen) Bergey *et al.*, *A. melanogenum* Beijerinck, *A. mobile* Tosic & Walker, *A. orleanense* Henneberg, *A. oxydans* (Henneberg) Bergey *et al.*, *A. pasteurianum* (Hansen) Beijerinck, *A. peroxydans* Visser't Hooft, *A. rancens* Beijerinck, *A. suboxydans* Kluyver & de Leeuw, *A. turbidans* Cosbie, Tosic & Walker, *A. viscosum* (Day & Baker) Shimwell and *A. xylinum* (Brown) Holland. These were obtained from the following sources: National Collection of Type Cultures, Lister Institute, England; Dr K. B. Raper, North Regional Research Laboratory, Peoria, Illinois, U.S.A.; and Prof. A. J. Kluyver, Delft, Holland.

The description of this organism was also compared with such information concerning *Acetobacter* species as is available in the literature (Breed *et al.*, 1948; Henneberg, 1926; Jørgensen, 1948). On this evidence it was concluded that *A. capsulatum*, first described by Shimwell (1936), is the only *Acetobacter* species which is somewhat similar to the one now described. Nevertheless, the new organism differs from *A. capsulatum* in such a number of cultural, physiological and biochemical characters that we consider it to be a new *Acetobacter* species. For example, *A. capsulatum* forms acid from glycerol, L-arabinose, D-galactose, maltose, mannitol, and salicin, whereas the new organism is unable to form acid from any one of these substances. *A. capsulatum* also forms dihydroxyacetone in a glycerol medium and the new organism has not shown this behaviour. Also, the giant colonies of the new bacterium are readily distinguishable from those of *A. capsulatum*. Moreover, the new species is the only *Acetobacter* so far isolated and studied which forms a starch-like polysaccharide, in contrast

to all other authentic *Acetobacter* species, which are unable to form this substance under comparable conditions. The ability of the new species to form much viscid material in malt extract media of low pH value suggested the name *A. acidum-polymyxa* and, up to the date of the present communication, the authors have used this name provisionally to distinguish the organism from others during laboratory studies of *Acetobacter* species. In conformity with the recommendation of the International Bacteriological Code of Nomenclature, not to coin names from words of different languages, the provisional specific epithet is now replaced by the all-Latin alternative 'acidum-mucosum'. The species *A. acidum-mucosum* is now formally proposed as new.

Acetobacter acidum-mucosum Tosic & Walker, n.sp.

Cells ovoid or short rods, $0{\cdot}6$–$0{\cdot}8\,\mu. \times 0{\cdot}7$–$1{\cdot}4\,\mu$. Non-motile. No endospores. Capsules present, the size depending on the pH of the medium; at pH 4·5 or lower $3{\cdot}2 \times 4{\cdot}8\,\mu.$, some even up to $8\,\mu.$ in length; at pH 4·9 or higher $1{\cdot}6 \times 2{\cdot}0\,\mu$. Giant colony convex, semitranslucent, very shiny.

Aerobic. Optimum temperature, 25–30°. Optimum hydrogen ion concentration, pH 3·7–6·0.

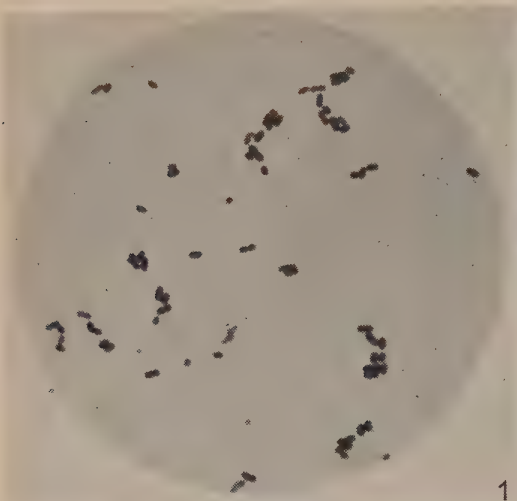
Rapid growth in malt extract medium with bluish ascending film, pronounced turbidity and powdery deposit. Growth strong in beer with production of turbidity and a viscid deposit. Growth in nutrient broth slower and more delicate than in beer or in malt extract.

Rapidly oxidizes ethanol to acetic acid. Acid from propanol, ethyleneglycol, D-xylose and D-glucose. No acid from glycerol, L-arabinose, D-fructose, D-galactose, sucrose, maltose, lactose, mannitol, salicin and dextrin. Unable to utilize ammonium salts as sole source of nitrogen; slowly oxidizes acetic acid in a yeast-water medium; non-ketogenic; on malt agar forms a substance which gives reactions for starch.

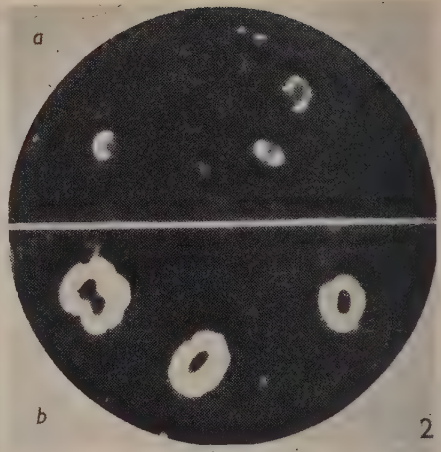
Isolated as a contaminant of a brewery 'top-yeast' (*Saccharomyces cerevisiae*), Manchester, England, April 1940.

Sub-cultures from the type culture have been deposited at the National Collection of Type Cultures, Colindale, London (Culture No. 6429), the North Regional Research Laboratory, Peoria, Illinois, U.S.A., the Laboratory of Prof. A. J. Kluyver, Delft, Holland, the Laboratory of Prof. M. Stacey, University of Birmingham, the Wellcome Research Laboratories, Beckenham, Kent, and with the authors at the Medical Research Council Unit for Chemical Microbiology, University of Cambridge (J. T.) and the Faculty of Technology, University of Manchester (T. K. W.).

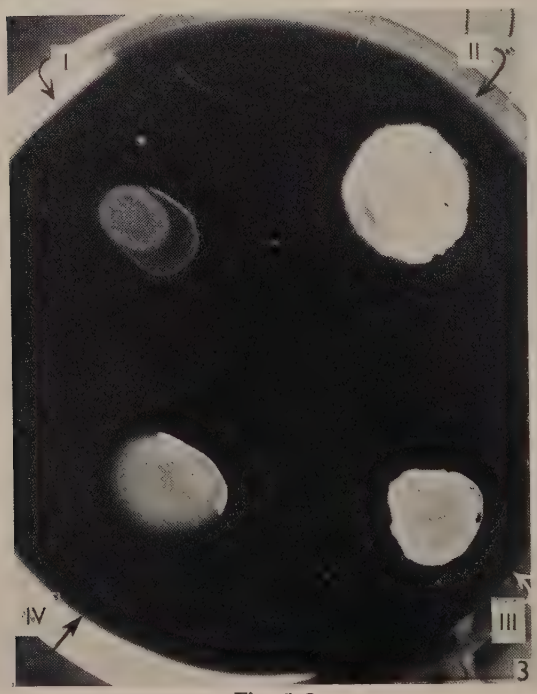
We should like to acknowledge valuable assistance from Dr Dora Kulka on a number of occasions, and to express our thanks to Mr W. C. MacLeod for taking the photomicrographs reproduced in Pl. 1, fig. 2.



1



2



3

Figs. 1–3

J. Tosic & T. K. Walker—*Acetobacter acidum-mucosum* n.sp. Plate 1

REFERENCES

Baker, S. L. (1920). Technique for the demonstration of the capsules of bacteria. *Brit. J. exp. Path.* **1**, 127.

Breed, R. S., Murray, E. G. D. & Hitchens, A. P. (1948). *Bergey's Manual of Determinative Bacteriology*, 6th ed. London: Baillière, Tindall and Cox.

Henneberg, W. (1926). *Handbuch der Garungsbakteriologie.* Berlin: Parey.

Jørgensen, A. (1948). *Micro-organisms and Fermentation*, 7th ed. London: Griffin.

Kulka, D., Preston, J. M. & Walker, T. K. (1949). Giant colonies of *Acetobacter* species as an aid to identification. *J. Inst. Brew.* **55**, 141.

Shimwell, J. L. (1936). Study of a new species of *Acetobacter* (*A. capsulatum*) producing ropiness in beer and beer-wort. *J. Inst. Brew.* **42**, 585.

Tosic, J. & Walker, T. K. (1946). Procedure for the characterisation of the acetic acid bacteria. Parts I and II. *J. Soc. chem. Ind., Lond.*, **65**, 104 and 180.

Walker, T. K. & Tosic, J. (1942). *Acetobacter* species as common contaminants of brewery yeast. *Biochem. J.* **36**, vii.

EXPLANATION OF PLATE

Fig. 1. Cells of *Acetobacter acidum-mucosum* n.sp. grown on malt extract agar at 30° for 48 hr. (×1200.)

Fig. 2. Cells of *A. acidum-mucosum* grown in malt extract medium at 30° for 10 days, showing capsules formed (*a*) at pH 7·0, and (*b*) at pH 4·0. (×2100.)

Fig. 3. Giant colonies of *Acetobacter* spp., grown on malt extract agar at 17–21° for 14 days. Photographed with top lighting (×1). I, *A. suboxydans*; II, *A. acetigenum*; III, *A. acetosum*; IV, *A. acidum-mucosum.*

(*Received* 19 *July* 1949)

The Bacterial Genus *Lineola*

By E. G. PRINGSHEIM
Botany School, University of Cambridge

SUMMARY: *Lineola*, a new bacterial genus, is characterized by large and very long cells of an unusual type and by the formation of motile trichomes of considerable length which may be branched.

Two species were isolated in pure culture by the use of capillary pipettes. Even when their nutritional requirements were known it was difficult to purify them by plating. The growths of the two species on agar are different because the trichomes of *L. articulata* are much softer than those of *L. longa*.

The cells of both species contain a large number of nucleoids and divide by constriction. In *L. longa* the daughter cells remain firmly connected, whereas in *L. articulata* the links become pliable, the whole chain-like trichome bending when swimming. Both species are Gram-negative and have numerous flagella. No spores were found.

Media with peptone, meat extract, yeast extract, and the like must be supplemented with acetate and with agar or certain other substances, to support multiplication.

In a previous paper (Pringsheim & Robinow, 1947) mention was made of a bacterium which was provisionally named *Lineola longa* because O. F. Müller had observed a *Vibrio lineola* possibly identical with the newly isolated form. From Müller's figures (1786, pl. VI) it was clear that his organism was not the same. Since no indication could be found that *Lineola longa* had previously been described, and as the generic name *Lineola* has never been used, this species is now proposed as the type of a new genus.

In addition, a second species was isolated for which the name *L. articulata* is proposed. Other, smaller, species exist which can probably be included in this genus. One with slightly pointed ends and only 1·0–1·2 μ. wide was recognizable. It is, however, the great size of the cells which gives interest to the two species described here. They may, like *Caryophanon* (Peshkoff, 1940; Pringsheim & Robinow, 1947), be useful for investigations on cell-physiology, osmotic behaviour, and movement of bacteria. Cultures for investigations will be supplied on request and will be deposited with the American Type Culture Collection.

The two species of *Lineola* are found in decaying plant material from quiet waters, *L. longa* also in infusions of fresh cow-dung. It is surprising that they have never attracted attention, but they are not mentioned in Migula's *System der Bakterien* (1900) nor in *Bergey's Manual* (1939, 1948). This omission is probably due to the difficulty in obtaining pure cultures of these organisms by ordinary bacteriological methods.

Isolation

L. longa, although observed previously on several occasions, was first recognized in infusions of cow-dung from Cambridge in October 1945. It attracted attention by the movements of its trichomes. These are sufficiently

large to be sucked into capillary pipettes under a dissecting microscope and freed from contaminants by three transfers to sterile washing fluids (Pringsheim, 1937, 1946). The first pure culture was obtained in cow-dung extract supplemented with 0·2 % meat extract. When its requirements were better understood it was possible to obtain isolated colonies of another strain on agar plates by streaking out from a dense population in polluted water. After 2 days at room temperature the large colonies could be recognized and were sufficiently distinct from other growths to be isolated with the help of a capillary pipette.

L. articulata was detected in September 1947 under similar circumstances in an infusion of decaying plant material from the New Forest. My attention was drawn to it by the flexibility of the long chains of rods as they swam through the water. This species was also seen several times subsequently in similar micro-communities. Isolation was performed in much the same way as with *L. longa*. Purification by plating was not successful. Its requirements for growth are similar to those of the other species, except that it develops more readily in liquid media and is not so liable to lose its motility.

Cultural appearances

Both species of *Lineola* form chains of rods of such width that the growths can be recognized as filamentous with a strong hand lens. Colonies on agar are, however, different and characteristic in the two species. It is several days before single trichomes attain sufficient size to become visible to the naked eye.

Large colonies of *L. longa* on agar are flat, irregularly shaped semi-confluent patches with fringes at the edges and are composed of long trichomes (Fig. 1*a*, *b*) as in other filamentous bacteria (Bisset, 1939). Single organisms give rise to small medusa-head colonies. When they have grown larger they are bluish white when viewed against a dark background and show a watered silk effect. The organisms cohere so that they must be removed almost completely as a colony for transfer to a fresh medium. If the colony is only touched with the needle, nothing may adhere, and subcultures are sterile. The appearance of the growth is evidently influenced mainly by the length and rigidity of the trichomes.

L. articulata does not produce fringed colonies because of the flexibility of the filaments which are kept together by the surface tension of the water-film covering the colonies on agar. Large colonies are wavy or lobed in outline and curly in structure (Fig. 2). They are more like mother-of-pearl than watered silk, and are iridescent rather than bluish. *L. articulata* is less coherent than *L. longa* and more readily transferable.

The youngest growths of the two species on agar are as different as are the older ones. Viewed by low power 1 day after inoculation *L. longa* appears as more or less parallel and straight threads. By elongating and breaking up into shorter lengths the trichomes soon form bundles (Fig. 3*a*). Isolated trichomes form, by intercalary elongation, coiled aggregations at intervals, connected by almost straight sections (Fig. 3*b*). Still later a maze of curved filaments is formed so that the initial arrangement can no longer be recognized.

Young growths of *L. articulata* appear as irregularly undulating snake-like threads which develop into small, narrow elongated colonies. These later

become V-, Y-, or X-shaped and develop into increasingly regular patches, at first still showing points and outgrowths which, however, change later into spiral areas within the large colonies (Fig. 4*a*, *b*).

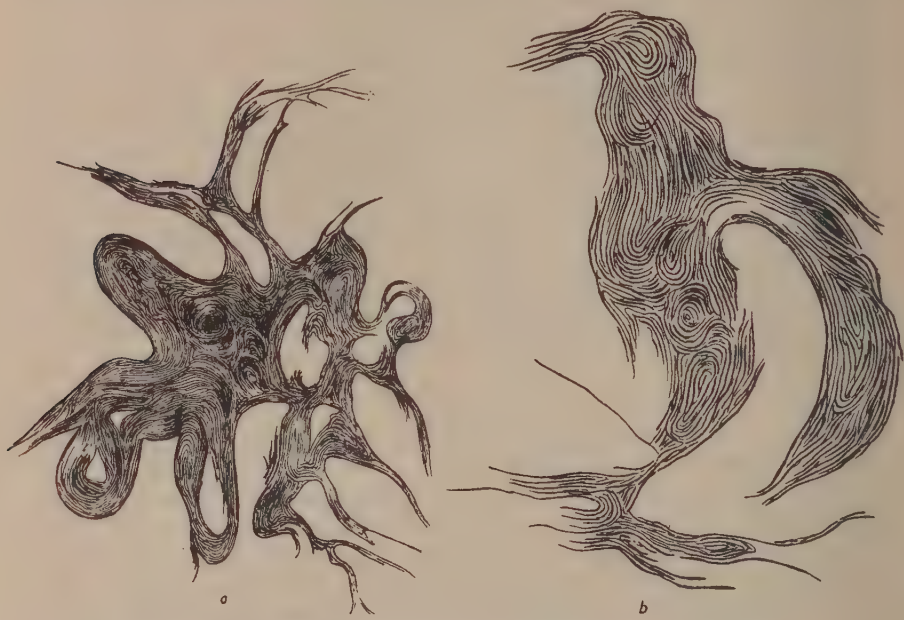


Fig. 1*a*, *b*. Colonies of *L. longa* on acetate peptone yeast extract agar; (*a*) ×25; (*b*) detail. ×50.

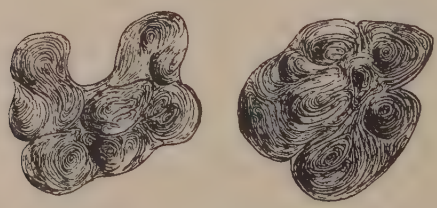


Fig. 2. Colonies of *L. articulata* on acetate peptone yeast extract agar. ×25.

Whereas both species grow abundantly in the condensation fluid of agar slopes, their multiplication in liquid media occurs only in the presence of certain combinations of substances. At first multiplication was obtained only when cow-dung extract was added to a medium on which growth could otherwise be secured only in combination with agar. Later cow-dung was found to be replaceable by soil extract or dilute sea water, though not quite so effectively with *L. longa* as with *L. articulata*. The addition of very small amounts of agar (0·05–0·2 %) proved also to be beneficial. Even distilled water in which agar had

been soaked, added to a medium which without agar did not support growth, permitted the development of both species of *Lineola*.

In all liquid media growth of *L. longa* in a non-motile state is best near the surface, although when slight or in the early stages, it may start near the bottom. If it is not too meagre a ring is formed just below the meniscus,

Fig. 3*a,b*. Young colonies of *L. longa*; (*a*) after 1 day: trichomes still straight; (*b*) after 2 days: elongating filaments curling up. × 25.

Fig. 4*a,b*. Young colonies of *L. articulata*; (*a*) after 1 day: snake-shaped owing to softness of trichomes; (*b*) after 2 days: trichomes sliding along one another during elongation and, when frictional resistance increases, curling in the middle of growing colonies. × 25.

which becomes detached and falls to the bottom on the slightest disturbance. When growth is rapid and motility strong, *L. longa* produces a nearly homogeneous turbidity. In less favourable conditions motility is diminished and the trichomes are twisted and entangled, so that their aggregates can be seen with the naked eye as silky white floccules. Sometimes filaments of enormous length develop, eventually forming deposits like cotton-wool on the bottom of the tube.

L. articulata, which is rarely non-motile, fills the whole fluid with evenly distributed trichomes. These are seen with a hand lens to swim in all directions at random, never aggregating and only settling on the bottom in ageing cultures.

In agar stab cultures growth of both species develops only on or very near the surface. This is true even with not more than 0·25 % agar which suffices to prevent the inoculum from falling to the bottom or moving about.

Morphology and movement

Lineola longa

Under natural conditions and in favourable media *L. longa* appears in the form of long, slightly and irregularly curved trichomes, 1·4–1·6 μ. in diameter and of great length. Free-swimming trichomes may be more than 200 μ. long; the longer they are, the less regular and lively is the movement. Even the longest ones are relatively rigid, although a slight pliability and elasticity can be discerned when the trichomes encounter mechanical obstruction.

Fig. 5. Growing motile trichomes of *L. longa* after fixation with osmic acid: division by constriction and fission. ×750.

All but the shortest trichomes are subdivided by constrictions which accentuate the demarcations between the rod-like sections but do not at first truly divide the interior of the cells. Later they are completed to become cross-walls (Fig. 5). These can be demonstrated with tannic acid-crystal violet (Pringsheim & Robinow, 1947, p. 276 and pl. 5, fig. 16). By Giemsa-staining it can be shown that these rod-shaped sections are not further subdivided; they thus form cells, 25–40 μ. long, only a relatively small number of which constitute even the longest trichomes. As each rod-shaped section contains a considerable number of Feulgen-positive bodies, presumably of a nuclear nature, the sections are comparable to multinucleate cells or coenocysts of algae.

Young trichomes, 50–60 μ. long, begin first to show a slight constriction at about the centre. As the whole trichome grows longer the constrictions become narrower, while new ones appear in the two halves, though generally not at regular intervals. The majority of trichomes in a healthily growing culture are composed of four incompletely separated sections; when the first constriction is completed by the formation of a cross-wall the filament divides into two by transverse fission at this place.

Strangely enough the trichomes are sometimes branched, this phenomenon being quite frequent at times. Sometimes branched forms are composed of only three rods. When the main trichome was considerably longer than the side-branch the movement was almost normal, in which case the branch rotated with the entire structure. More fully developed branches rendered the movement irregular, so that progress became almost impossible. The cells were plasmolysed in the normal way in hypertonic solutions (cf. p. 206).

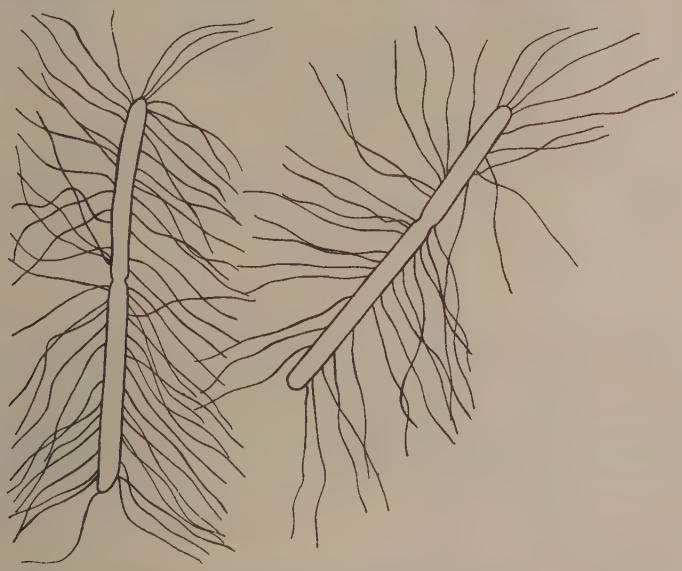


Fig. 6. ***L. articulata*** **from agar slope; flagellar staining, preparation by Dr W. J. Dowson. ×2000.**

L. longa stains like other bacteria. It is Gram-negative and when in a healthy state does not contain appreciable amounts of granular material; these, however, accumulate in old cultures. It is motile by a large number of peritrichously arranged flagella. They are evenly distributed over the whole of the trichomes. No mucilage or sheath could be demonstrated. When a filament is torn, the remainder of the longitudinal cell-walls have the appearance of a sheath, but on closer inspection their true nature is seen. The bacterium is fairly constant in size, shape and reaction to cultural conditions. No difference between various types of colonies, e.g. roughness and smoothness, was observed.

Lineola articulata

This species resembles *L. longa* in many features. The cells are 1·4–1·6 μ. wide and 30–50 μ. long. They stain and are covered with flagella much in the same manner as *L. longa* (Fig. 6). The elongation, division by constriction and subsequent separation of the cells are also the same.

The distinctive character is the pliability of the connecting links, once the cross-walls separating the cells have been completed. How the cells remain attached is not clear. Narrow gaps may be seen between them, so that they are apparently unconnected, although linked up to form tough coherent trichomes (Fig. 7) which never break during movement, not even when a trichome is sucked into a capillary and blown out again. The joints must therefore have a remarkable toughness, in spite of being much more pliable than the inflexible cells. At the same time they possess a certain degree of rigidity since, during forward movement, the same slight curvatures often reappear again and again after each rotation (Fig. 8*d*).

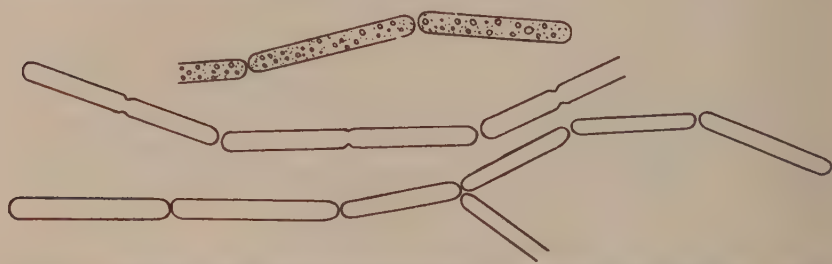


Fig. 7. *L. articulata*, cells with granules, trichome with constrictions, and branching. × 2000.

No material filling the apparent gaps nor any sheath bridging them could be detected by staining with methylene blue, safranin or congo-red, nor by means of India ink and nigrosin, in living or in dried preparations. Dark-ground illumination was similarly unsuccessful. It is difficult to imagine any substance tough enough to hold the sections together and yet so watery that India ink and nigrosin could penetrate. No evidence of linkage by means of delicate threads or plasmodesmata could be found.

Branching is more frequent in *L. articulata* than in *L. longa*. Side branches are attached to the main trichome in the same indefinable manner as the sections of the original trichome, i.e. seemingly unconnected and yet held in their places in spite of vigorous movement.

Free-swimming trichomes consist generally of a greater number of cells than those of *L. longa*. Often sixteen of them are found linked together, but irregularities in cell-division occur, so that the number, after four divisions, is usually less. In any population there are short trichomes also, consisting of two or more cells which eventually grow into long ones.

When undisturbed most of the filaments swim over a limited distance in a nearly straight line, so that it is clear that the flagella are well synchronized. A few trichomes, and many more after agitation, assume a more or less curved shape. They may swim in a circular course, curling up to form spirals, or they become horse-shoe shaped, progressing for a little while with the convexity in front or continually changing their direction, until one of the arms prevails and pulls the rest along so that the whole trichome becomes straight again. Simple filaments move and curve gracefully in a manner reminiscent of railway trains in a marshalling yard (Fig. 8*a–c*).

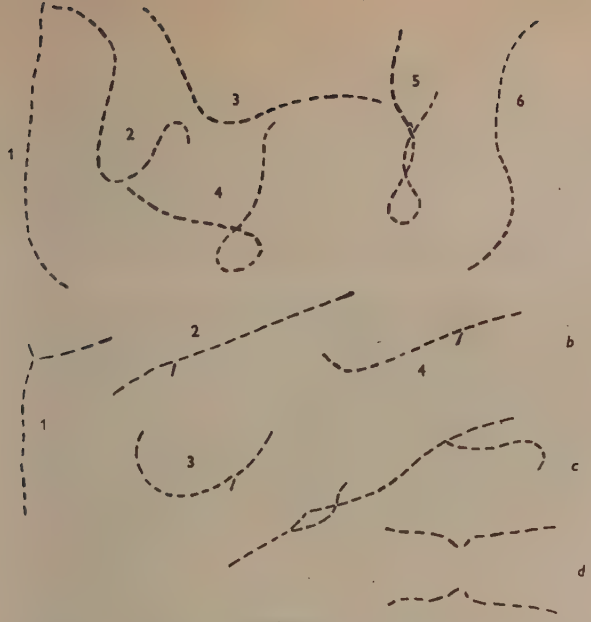


Fig. 8

Fig. 9

Fig. 8. *L. articulata*, schematic sketches of moving trichomes. (*a*) 1–6: stages in the movement of a trichome; (*b*) 1–4: the same, depicting trichome with one unicellular branch attached; (*c*) motile trichome with two multicellular branches; (*d*) two stages of a rotating trichome with characteristic curvature, given as if it had not moved forward meanwhile, in order to show the symmetry of the two appearances. The unaltered shape indicates a marked rigidity in spite of the pliability often exhibited. ×50.

Fig. 9*a*,*b*. *L. articulata*: plasmolysis; (*a*) artificial sea water of double concentration; (*b*) 5 % glucose solution.

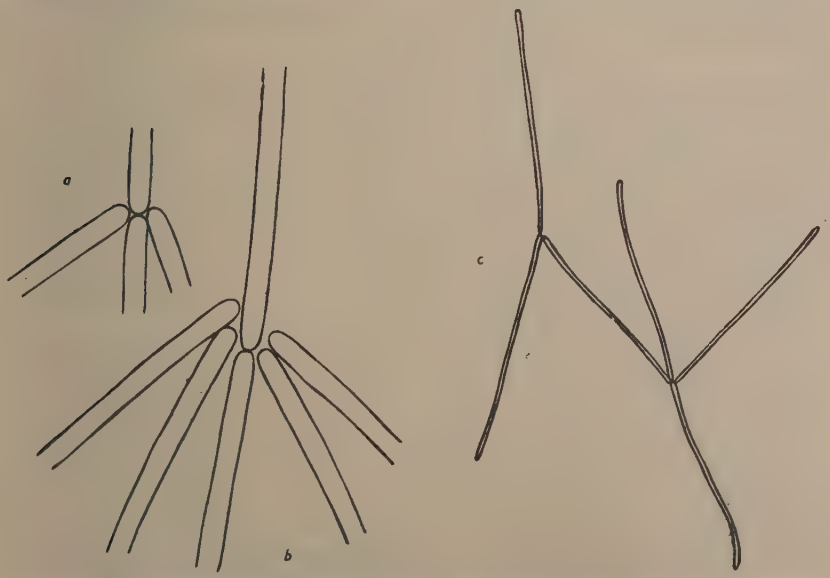


Fig. 10*a*–*c*. *L. longa* branched trichome. (*a*) two cells attached to a joint; (*b*) four cells near to one joint; (*c*) complex aggregation of cells. (*a*) and (*b*), ×2000; (*c*) ×400.

From the appearance and variation of these movements it is clear that they are caused by the action of the flagella. The individual cells do not change shape while rotating. This is best seen when there are slight irregularities in their form. When the larger part of a trichome drags along a portion bent in another direction obviously struggling to have its own way, one can watch the action of the conflicting forces and their influence in decreasing the speed and regularity of progress.

An interesting comparison is provided by *Bactoscilla flexibilis* (Pringsheim, 1949, p. 72), belonging to the Vitreoscillaceae, which are characterized by their gliding movements, combined with bacterium-like shape and size of cells. Its rod-shaped sections are linked together, so that during movement the trichome bends at the joints between the rods. The connecting matter is invisible, and the rods themselves remain straight. The movement, however, unlike that of *Lineola articulata*, is not caused by flagella; the trichomes adhere. at least partly, to the substrate while they glide along, and the joints appear to bend actively, not passively.

A further significant difference between the organization of *Lineola* and that of the gliding organisms is the osmotic behaviour of the cells. Whereas in Myxophyceae, Beggiatoaceae and Vitreoscillaceae (Pringsheim, 1949) the cells shrink as a whole in hypertonic solutions, plasmolysis can be beautifully demonstrated in *L. longa* and in *L. articulata* (cf. p. 203). In sea water, for instance, and in glucose solutions the cytoplasm retreats from the cell-wall in many places leaving more or less hemispherical cavities filled with the plasmolysing fluid (Fig. 9*a*, *b*). The cell-walls remain in about the same position and are clearly seen, while nothing of the kind could be detected in similarly treated trichomes of *Vitreoscilla moniliformis*, the largest of the species of this colourless gliding genus.

Branching

Branching occurs in both species, more frequently in *Lineola articulata* than in *L. longa*. The incessant movement made it impossible to investigate the origin of the branches. Their development could only be interpreted on the ground of their appearance. The use of fixatives and stains was ineffective. On application of osmic acid vapour, formalin and mercuric chloride the trichomes are disrupted into single cells or pairs. Direct addition of osmic acid solution caused the movement to cease while many trichomes were still coherent, but a real attachment becomes obvious only when the trichomes are in motion. Various vital stains were tried without useful results.

Most branches consist of single rods or short chains but, especially in *L. articulata*, longer side branches are not uncommon so that it was sometimes impossible to tell which was the main trichome and which the branch. In *L. longa* two, three or even more cells may be attached to the main axis near to one another (Fig. 10), while in *L. articulata* more than two were not found at one joint.

The branches are almost always attached where the cells meet. Only when more than two are close together, the odd ones are shifted slightly to the side. As the cells divide by transverse fission and not longitudinally, the

phenomenon of branching is difficult to explain. The habitual insertion of side branches at the joints is scarcely compatible with 'false branching' as it is found in Myxophyceae and in *Cladothrix*. No stages were found which could be interpreted this way.

There remains the possibility that previously independent cells settle on other trichomes. This interpretation cannot readily be accepted because these bacteria are never found attached to other bodies, but certain observations are in its favour. The predilection for the joints would, of course, require to be explained by special properties they possess. Chemotaxis is not probable. It is, however, at least feasible that the connexion between the rods is effected by some adhesive substance to which other cells may also stick, although in rare instances cells and cell-chains were found attached near the middle of a cell, so that it would be necessary to assume that the cell ends retain their adhesive nature after separation. This is not very probable since they do not stick to other bodies. Moreover, particles of India ink, although sometimes adhering to the organisms, did so completely at random without any preference for the ends or joints. The problem therefore remained unsolved. The actual process of attachment also could not be watched; but two or three cases were observed where a side-branch became detached.

Comparison with other bacteria

The organization of *Lineola*, characterized by the very long multinucleate cells, the contractions which mark far in advance the places of division, and the formation of consistently motile trichomes, may in the first instance appear curiously different from that of other bacteria, just as in another way do those of *Caryophanon* (Peshkoff, 1940; Pringsheim & Robinow, 1947) and *Oscillospira* (Pringsheim, 1949, pp. 72, 99). It is therefore necessary to discuss the differences and similarities between *Lineola* and other bacteria.

To begin with the largest forms, the shape of the cells of *Caryophanon* and *Oscillospira* is of course very different, these two genera having short discoid cells. Similarity is, however, found in the early accentuation by constriction of the places where separation will later take place. A difference is again found in the sections of *Caryophanon* and *Oscillospira* between two constrictions being composed of a number of serially arranged cells, whereas those of *Lineola* are unicellular, cross-walls not being formed between the nucleoid structures. Similar differences are found in certain groups of algae, for example, the Chlorophyceae, where the species *Ulothrix* and *Cladophora* represent organizations somewhat parallel to *Caryophanon* and *Lineola* respectively.

As long as the rods of well-known bacteria were mistaken for cells, the difference between them and *Caryophanon* was considerable, but since we know that they are in most cases composed of several cells (Robinow, 1945; Pringsheim & Robinow, 1947, p. 272, fig. 1), the division of their rods is comparable with the constriction and separation of the sections of *Caryophanon* and *Lineola*. The latter differs mainly in the lack of septa dividing the multinucleate sections into cells. Except for the nucleoids, *Bacillus anthracis* resembles *Lineola* in its cylindrical cells (Flewitt, 1948, pl. 1, fig. 4), whereas

Corynebacterium is more like *Caryophanon* in its shorter cells and their greater number in one rod (Bisset, 1949, p. 94, figs. 1, 2). Common to all these bacteria is the constriction, not immediately followed by division.

Nutrition

A medium containing 0·3 % yeast extract, 0·1 % meat extract, 0·1 % sodium acetate and 1·5 % agar was recommended in the previous paper as suitable for *Lineola longa*. No analysis of the exact nutritional requirements has yet been undertaken but, as far as is known, there are in this respect hardly any differences between the two species *longa* and *articulata*. A remarkable feature is the favourable effect of acetate, already found for *Caryophanon latum* which, however, can multiply in the absence of fatty acids, though neither species of *Lineola* can do so. The latter require even higher concentrations of acetate in order to thrive than are favourable for *Caryophanon*. When *L. longa* is transferred to the surface of an otherwise suitable agar medium but without acetate, only a very slight elongation of the trichomes takes place. When 0·1 % sodium acetate is added to the medium, the short trichomes grow out into long threads which, by intercalary elongation, are transformed into spirals (cf. p. 199).

Both species grow on agar media with acetate and meat extract or yeast extract, but much more profusely when peptone is added. An agar medium containing 0·2 % sodium acetate, 0·2 % yeast extract (Difco), and 0·2 % Bactotryptone with 1–1·5 % agar affords better growth than that given above. Strict neutrality as required by *Caryophanon* and other bacteria is not necessary. When the concentration of the nutrients is doubled no more multiplication occurs.

Neither glucose nor glycerol have any beneficial effect. Larger amounts of mineral salts, for example sea water, even diluted down to 1/4, are noxious. Growth is not appreciably quicker at 25–27° than at 18–20°. At 32° growth of *Lineola longa* is retarded, but *L. articulata* is still able to grow.

The results so far reported were obtained with agar-slope cultures. Liquid media proved more difficult to use, although both species are found in aqueous habitats. Dung extract and soil extract were suitable supplements to liquid media containing acetate, yeast extract and tryptone, which themselves could not support growth. Sea water diluted to 1/10 and Benecke solution were partially effective but not reliable, and agar in low concentrations was beneficial. *L. articulata* reacts more readily than *L. longa* to such additions. None of the growth factors contained in meat extract, yeast extract, liver extract, nor aneurin, can replace dung extract, so that the real nature of the relevant factor remains unknown.

DIAGNOSIS

Lineola Pringsheim, n.g.

Characterized by very long peritrichous rods, composed of one or two multinucleate cylindrical cells; non-sporing; Gram-negative. Forms motile often branched trichomes up to several hundred microns in length. The rods do not break up into smaller units at the end of the life cycle but divide into two by constriction and fission. Type species *L. longa*.

Lineola longa n.sp.

Cells 1·4–1·6 μ. wide and 10–50 μ., mostly 25–40 μ. long. Trichomes rigid, up to 300 μ. long. Non-motile trichomes of much greater length may be formed. Grows in pure culture on agar with yeast extract or meat extract, peptone, and acetate; in liquid media only when supplemented with extracts of dung or soil, or other growth-promoting substances. The type culture is that obtained from infusion of cow-dung from Cambridge on 25 October 1945. The subculture has been deposited with the American Type Culture Collection.

Lineola articulata n.sp.

Cells 1·4–1·6 μ. wide and 10–50 μ., mostly 30–40 μ. long. Motile trichomes up to 160 μ. in length with flexible joints connecting the individual rods.

Type culture obtained from water with plant débris from the New Forest, 30 September 1947. Nutrition similar to that of *L. longa*. Subculture deposited with the American Type Culture Collection.

Both species are frequently found in nature where plant material is decaying.

I wish to express my sincere thanks to Dr K. A. Bisset, Birmingham, for correcting the manuscript, and to Dr W. J. Dowson, Cambridge, for his help in flagella staining.

REFERENCES

Bergey's Manual of Determinative Bacteriology, 5th ed. 1939; 6th ed. 1948. London: Baillière, Tindall and Cox.

Bisset, K. A. (1939). The mode of growth of bacterial colonies. *J. Path. Bact.* **48**, 427.

Bisset, K. A. (1949). Observations upon the cytology of Corynebacteria and Mycobacteria. *J. gen. Microbiol.* **3**, 93.

Flewitt, T. H. (1948). Nuclear changes in *Bacillus anthracis* and their relation to variants. *J. gen. Microbiol.* **2**, 325.

Migula, W. (1900). *System der Bakterien.* II. *Specielle Systematik der Bakterien.* Jena.

Müller, O. F. (1786). *Animalcula Infusoria, fluviatilia et marina, etc.* Hauniae.

Peshkoff, M. A. (1940). Phylogenesis of new microbes, *Caryophanon latum* and *Caryophanon tenue*, organisms which are intermediate between blue-green algae and the bacteria. (Russian with English summary.) *J. gen. Biol.* **1**, 598.

Pringsheim, E. G. (1937). Beiträge zur Physiologie saprophytischer Algen und Flagellaten. I. *Chlorogonium* and *Hyalogonium*. *Planta*, **26**, 631.

Pringsheim, E. G. (1946). *Pure Cultures of Algae.* Cambridge University Press.

Pringsheim, E. G. (1949). The relationship between bacteria and Myxophyceae. *Bact. Rev.* **13**, 47.

Pringsheim, E. G. & Robinow, C. F. (1947). Observations on two very large bacteria, *Caryophanon latum* Peshkoff and *Lineola longa* (nom.provis.). *J. gen. Microbiol.* **1**, 267.

Robinow, C. F. (1945). Addendum to *The Bacterial Cell*, by R. J. Dubos. Cambridge, Mass.

(*Received* 26 *July* 1949)

The Mechanical Transmission and some Properties of Potato Paracrinkle Virus

By F. C. BAWDEN, B. KASSANIS and H. L. NIXON
Rothamsted Experimental Station, Harpenden, Hertfordshire

SUMMARY: With the aid of abrasives, paracrinkle virus, hitherto transmitted only by grafting, was transmitted to Arran Victory potatoes by inoculation with sap from infected plants, either symptomless King Edward or diseased Arran Victory. The proportion of plants that became infected was increased when they were kept in darkness for some days before inoculation.

Tomato plants were more readily infected than Arran Victory potatoes, no abrasive being needed. Infected tomatoes were symptomless, but electron microscopy showed their sap to contain rod-shaped particles of variable lengths and about 10 mμ. wide. Such particles have not been found in uninfected tomatoes and they are presumed to be the virus; they were destroyed by heating at 60°.

The nature of similar particles in King Edward and Arran Victory potatoes with paracrinkle is uncertain, because rod-shaped particles were also found in uninfected Arran Victory. Rod-shaped particles also occurred in uninfected plants of all other potato varieties examined and in newly raised potato seedlings; they were not transmitted, either by inoculation or by grafting, to tomato or other hosts tested, and they withstood heating to 98°.

These results with paracrinkle parallel those with certain other plant viruses. They invalidate theories that postulate its origin as a consequence of grafting and render unnecessary the concept that it is intrinsic to King Edward potatoes.

Salaman & Le Pelley (1930) gave the name paracrinkle to a disease which they found was produced when plants of certain potato varieties, particularly Arran Victory, were grafted with scions from apparently normal plants of the variety King Edward. They failed to cause paracrinkle by inoculating Arran Victory plants with sap from King Edward plants and attributed the disease to a graft-transmissible virus for which King Edward plants are symptomless carriers. Their original observations have been repeatedly confirmed and the many King Edward plants that have since been examined by grafting to Arran Victory have all been found to cause paracrinkle (Salaman, 1932; Barton-Wright & McBain, 1933). Indeed, although it is obviously impossible to prove, it seems highly probable that paracrinkle virus occurs in every plant of this clonal variety. Despite the fact that King Edward has been extensively grown for many years, and so has provided abundant sources of the virus, paracrinkle virus has not been recorded as occurring in any other potato variety in the field. It seemed reasonable to infer from this that the virus has no natural method of spread, which in turn implied that the virus was present in the original seedling from which the clone has developed, and that it has survived and multiplied only because its host is propagated vegetatively.

When first drawing attention to the inferences possible from the known occurrence of paracrinkle virus, Bawden (1939, p. 258) pointed out that, if the original King Edward seedling became infected from some external source, it seemed necessary to assume that the virus had some natural method of

spread which it has since lost. He suggested that it was equally plausible to assume that the virus had arisen *de novo* in the variety, but, as nothing definite is known about the origin or early treatment of the clone, stressed the speculative nature of any interpretations. Since then paracrinkle has figured prominently in discussions on the origins of viruses (Darlington, 1944, 1949; van der Plank, 1948), with statements becoming increasingly positive, although there has been little further work on the virus. That it is intrinsic to King Edward seems to have been accepted as established, and Darlington (1944), completely ignoring the fact that nothing is known about the chemical nature of paracrinkle virus, stated that 'What is a stable and presumably useful cell protein with one genotype acts as a destructive agent with another'. He also stated that 'the virus must have arisen by grafting' and claimed that it provided evidence that there is no essential difference between viruses and plasmagenes (the hypothetical units that determine cytoplasmic inheritance). King Edward rarely flowers and sets seed, so that the inheritance of paracrinkle virus is difficult to study on a large scale. However, Carson, Howard, Markham & Smith (1944) obtained twenty-two seedlings from the cross King Edward (female) by Flourball and found them all to be free from paracrinkle virus. These results are inadequate evidence that transmission through the egg never occurs, but at least show that such transmission can be regarded as exceptional rather than normal. Despite this difference between the behaviour of paracrinkle virus and a plasmagene, Darlington (1949) has continued to relate the two; to stress its origin from a normal plant constituent, he has separated it from other plant viruses, placing it in a group which he has called provirus, of which the distinguishing character is transmissible only by grafting.

Paracrinkle provides an excellent example of the manner in which theories can grow when they are untrammelled by facts, for the wealth of speculation about it is matched only by the poverty of knowledge. The work described in this paper was started to gain some information about the properties and transmission of this academically interesting virus, which also has the potentialities of a dangerous pathogen; it is still far from complete, but the results so far obtained suggest that at least some of the premises on which theories have been based are erroneous.

MATERIALS AND METHODS

The source of paracrinkle virus was provided by Stock Seed King Edward plants, which, from their appearance and the negative results of transmission tests to tobacco (*Nicotiana tabacum*, var. White Burley), *N. glutinosa*, *Datura stramonium* and tomato (*Lycopersicum esculentum*), var. Kondine Red, seemed to be free from all other known potato viruses. The presence of paracrinkle virus was readily demonstrated by grafting scions to Arran Victory potato plants, all of which developed symptoms. As noted by Salaman & Le Pelley (1930), the severity of symptoms varied widely in different plants, even when these were grafted with scions from the same King Edward plant. The new shoots of some plants developed symptoms within 10–12 days of grafting, the young leaves

showing a blotchy yellow mottle, becoming crinkled and deformed, and growing much more slowly than normal. In other plants, symptoms developed less rapidly and were less severe; growth was little checked, and the leaves showed various degrees of mottling and waving. Symptoms were more uniform in the progeny from these plants raised from tubers in the next year, but there was still some variation. Most were much dwarfed, with small and highly crinkled leaves. Some, however, were less severely affected and at first grew almost normally but later became mottled and crinkled. Similar variations sometimes occurred between individual shoots from one tuber, some being acutely affected and others almost normal.

Salaman (1932) noted such variations and interpreted them as showing that paracrinkle is caused by a complex of two viruses that occasionally disrupts, so that a shoot or plant contains only one. We have found no evidence to support this, for tubers from mildly affected plants have given rise to severely affected plants as often as not. This also suggests that the phenomenon does not occur because of the segregation of strains of paracrinkle virus that differ in virulence. No certain interpretation can be given, but it seems likely that symptoms reflect the degree to which the virus has invaded the tissues and multiplied in them. Thus, delay in the development of symptoms may mean incomplete systemic invasion, a phenomenon that also occurs sometimes with potato virus *Y*, shoots from infected tubers developing at first normally and virus-free and later becoming infected (Bawden & Kassanis, 1946).

Unless otherwise stated, inoculations were made by lightly dusting the leaves of test plants with either carborundum powder (400 mesh) or celite (a diatomaceous earth) and then rubbing them with the forefinger dipped in inoculum. The inoculum was juice obtained by macerating leaves in a pestle and mortar, and for paracrinkle virus usually came from severely diseased Arran Victory plants in their second or third year with paracrinkle, or from King Edward plants. Healthy potatoes of named varieties were all derived from the virus-free stocks selected at the Virus Research Station, Cambridge, and were kindly supplied by Dr Kenneth M. Smith. Unnamed varieties were supplied by Mr E. M. Hutton and were tuber progenies from seedlings bred in recent years. Current-year seedlings were from seed set by Majestic and Gladstone plants and supplied by Dr G. Cockerham. No known viruses were detected in any of these plants, although they were rigorously and frequently tested both by serological methods and by inoculation to such differential hosts as tobacco, tomato, *Nicotiana glutinosa* and *Datura stramonium*.

All specimens that were examined with the electron microscope were also tested by inoculation to this range of hosts, to ensure freedom from such viruses as potato *X*, *Y*, *A*, tobacco mosaic and cucumber mosaic. None of these was ever found, and we have no reason to suspect that any of our plants was infected with any known virus except paracrinkle. Clarified sap was used for preparing electron micrographs. Leaf laminae lightly sprinkled with Na_2HPO_4 were crushed with a pestle in a mortar, and the sap expressed through muslin. The sap was frozen overnight, thawed and clarified by centrifugation at 8000 r.p.m. Mounts for electron microscopy were made by placing small drops

of the clarified sap on the usual collodion-covered specimen grids; the drops were allowed to remain on the grid for about a minute, when the liquid was sucked off with a micropipette. The mounts were washed by dipping in distilled water, dried on hard filter-paper, and then shadowed with a 0·6–0·8 mμ. thickness of palladium, which was cast at an angle of about 12 degrees from the plane of the mount. The electron microscope was an R.C.A. model B, fitted with a three-electrode electron gun and used with an accelerating potential of 50 kV. Much of the work was solely visual, the searching of many fields to determine whether rod-shaped particles were present, and for this the extra illumination obtainable with the modified electron gun proved extremely valuable. Electron micrographs were usually taken at an instrumental magnification of about 10,000 times, the condenser being defocused to give the minimum brightness of illumination needed for visual focusing of the selected images.

RESULTS

Mechanical transmission of paracrinkle virus

Salaman & Le Pelley (1930) obtained no evidence that anything could be transmitted from apparently normal King Edward plants to other potato varieties or to tobacco and *D. stramonium*, by inoculation of expressed sap. So, too, with one exception, they failed to obtain transmissions from Arran Victory plants with paracrinkle. The one exception may have been a transmission of paracrinkle virus, but the results were equivocal as the inoculum also caused a mosaic in President potato plants, which carry paracrinkle, and gave a mosaic in *D. stramonium*, suggesting that there may have been a contamination with a strain of potato virus *X*. Since their work, it has been found that the transmissibility of some viruses by inoculation is greatly facilitated by incorporating certain abrasives in the inocula (Rawlins & Tompkins, 1936; Kalmus & Kassanis, 1945), and by preconditioning the host plants by keeping them under reduced illumination (Bawden & Roberts, 1947, 1948). Thus it seemed worth re-examining the possibilities of transmitting paracrinkle virus by mechanical inoculation with sap.

A preliminary test in 1948 suggested that this could be done, although not readily. Sap from an Arran Victory plant with paracrinkle was inoculated to ten healthy plants, one of which developed a mild blotchy mottle. The symptoms were less severe than those in grafted plants, but when the tubers were planted in 1949 they gave rise to plants with characteristic reactions. There was some difference in the severity of symptoms in individual plants, particularly early in the season, but no more than occurs with the progeny of plants infected by grafting.

In 1949 more extensive tests were made. In March, thirty healthy Arran Victory plants were inoculated with sap from a paracrinkle plant; twenty had been kept under normal glasshouse conditions and ten had been kept in darkness for the 4 days immediately before they were inoculated. Two plants in each set developed a blotchy mottling and showed some ruffling and waving of the leaves (Pl. 1, fig. 1). Other plants, however, had become infected, but had

shown no symptoms. This was demonstrated by harvesting the tubers in June, breaking their dormancy and planting them again, when, in addition to the progeny from the four plants that had previously shown symptoms, progeny from six others, four of which were from plants placed in the dark before inoculation, showed unmistakable symptoms of paracrinkle. Comparable results were obtained in a second experiment in April with thirty Arran Victory plants, fifteen of which were placed in the dark for 3 days before being inoculated with sap from a paracrinkle Arran Victory. Five of this fifteen developed symptoms within a fortnight of inoculation, whereas all those not placed in the dark remained apparently healthy. Again more infections had occurred than had appeared, and when tubers were replanted, those from six plants that had been kept in the dark, and from two that had not, produced plants with characteristic symptoms. Thus, of a total of sixty Arran Victory plants inoculated, at least eighteen became infected, six of the thirty-five kept under normal glasshouse conditions and twelve of the twenty-five placed in the dark for some days immediately before inoculation.

Sap from diseased Arran Victory plants was used as the inoculum for these tests, on the assumption that it might contain more virus than sap from King Edward plants. This assumption may well have been wrong, for of three Arran Victory plants that were inoculated in May with sap from a King Edward, two developed symptoms of paracrinkle. Later tests, in which tomato plants were inoculated, also suggested that King Edward plants are as good a source of virus as diseased Arran Victory. Although 100 % transmission was not obtained in the inoculation tests with potatoes, the results were adequate to show that graft-transmission is not obligatory for paracrinkle virus; it seems that previous failures occurred because the virus content of sap is low, below that needed to cause infection when potatoes are simply rubbed or scratched with sap, and is about the minimum infecting dose with such additional aids as suitable abrasives and lowering the resistance of plants by keeping them in the dark. Paracrinkle virus is not unique in this respect; potato virus *A*, for example, has been transmitted by inoculation from infected to healthy potato plants only with the aid of an abrasive (Bawden, 1936). Paracrinkle and potato virus *A* also seem to resemble one another in being more readily transmitted by inoculation to certain other hosts than to potato.

The sap from diseased Arran Victory plants, used as inoculum in the experiments already described, was inoculated with the aid of celite to five plants each of tobacco, tomato and *D. stramonium*, partly to ensure its freedom from potato virus *X* or other possible contaminants and partly to see whether these species could be infected. None of them developed any symptoms, but a month later scions were taken from them and grafted on to healthy Arran Victory plants. All the plants grafted with tobacco and *D. stramonium* scions remained healthy, whereas the five grafted with tomato scions developed paracrinkle (Pl. 1, fig. 2). The fact that the tomato is a symptomless carrier of paracrinkle virus and is more readily infected than potato by inoculation was confirmed in other tests. In one test, four different inocula were used, namely, sap from either King Edward or diseased Arran Victory, each with and without celite. None

of the tomato plants showed any obvious symptoms, but that infections had occurred was shown when one plant from each treatment was grafted to a healthy Arran Victory plant, for all of the grafted plants developed symptoms of paracrinkle. Only one plant from each treatment could be tested in this manner as we had only four remaining Arran Victory plants, but examination with the electron microscope of clarified sap from the inoculated tomatoes suggested that all of them had become infected. Thus, the virus can be transmitted to tomato from potato without the aid of an abrasive, in the same manner as potato virus *A* can be transmitted to tobacco (Bawden, 1936). Other results from electron microscopy also suggest that the virus is readily transmissible from infected to uninfected tomato plants by inoculation of sap without using any abrasive; with the aid of celite, we have produced infection using tomato sap diluted 1 in 1000.

Rod-shaped particles in infected and uninfected potatoes

When examined with the electron microscope, clarified sap from King Edward plants and from Arran Victory with paracrinkle was found to contain rod-like particles of varying lengths but a constant width of about 10 mμ. (Pl. 1, fig. 3). In other solanaceous species examined, for example, tobacco, tomato, *D. stramonium* and *Nicotiana glutinosa*, similar particles were found in plants infected separately with a number of different viruses, but not in uninfected seedlings. Their relevance to paracrinkle virus, however, was rendered uncertain by the fact that apparently similar, though fewer, particles occurred in clarified sap from uninfected Arran Victory plants (Pl. 1, fig. 4). Examination of sap from the five plants each of tomato, tobacco and *Datura stramonium*, already mentioned as having been inoculated with paracrinkle virus and grafted back to healthy Arran Victory, showed that some rod-like particles could be transferred from potato to tomato plants. All five tomato plants contained rod-like particles indistinguishable from those in the potato (Pl. 1, fig. 5), whereas no such particles were found in any of the tobacco or *D. stramonium* plants. As only the tomato plants produced paracrinkle when grafted to Arran Victory, this suggested a connexion between the rods and the virus, though it was inconclusive, for the rod-shaped particles present in uninfected potatoes might also be transmissible to tomato, but not to tobacco and *D. stramonium*.

Further studies were therefore made to determine the occurrence and properties of such particles in normal potato plants. In addition to Arran Victory, sap from the varieties Arran Banner, Arran Pilot, British Queen, Craig's Defiance, Great Scot, Katahdin and Kerr's Pink, and from two of Hutton's new varieties, numbers 11–76 and 11–84, was examined with the electron microscope, and similar rod-shaped particles were found in all. Their occurrence in the last two suggested that the rods are normal constituents of potato leaves, and not viruses which the varieties might have contracted from external sources and be carrying symptomlessly. Support for this interpretation was provided by raising seedlings, from seed set by Majestic and Gladstone, in insect-proofed glasshouses, conditions that preclude natural infections with

most viruses. Sap was taken from six such seedlings when they were about 4 in. high, and similar rod-shaped particles were found in all. Preliminary tests suggest that more of these rods occur in extracts of macerated petioles than of leaf laminae.

Tests were next made to determine whether these particles from uninfected potato plants could be transmitted to other species. Sap from all the varieties and the current-year seedlings was inoculated to tobacco, tomato and *Nicotiana glutinosa* plants, and scions from each variety were also grafted on to tomato plants. A month later, sap from the inoculated and grafted plants was examined with the electron microscope, and with one exception no rods were found in any. The exception was one mount from the tomato plant grafted with 11–84, in which two rods only were seen. Further preparations were made from this plant, but no more rods were seen. No positive interpretation of this one result can be given; it is unlikely that the plant had become accidentally infected with a rod-shaped virus, for had this happened later examinations should have shown more rods, and the test plants inoculated with the preparation all remained healthy. The rods may have been bacterial flagella or some other material accidentally introduced during the preparation of the mount. These negative results from control potatoes contrast sharply with those obtained when similar transmissions were made from King Edward or from Arran Victory plants infected with paracrinkle virus, for then rod-shaped particles were readily and regularly found in every tomato plant. This suggests strongly that the rods found in the inoculated tomato plants are paracrinkle virus, though this cannot be demonstrated conclusively. In this connexion, it is significant, too, that more rods occur in sap from leaf laminae of King Edward and from diseased Arran Victory plants than in sap from other varieties or from healthy Arran Victory, although accurate quantitative studies are impossible. The fact that fewer rods were found in sap from Arran Victory leaves that showed slight symptoms of paracrinkle than from those that were severely diseased, also suggests a connexion with the virus, and that variations in symptoms reflect differences in the extent to which the virus has multiplied and become fully systemic. Further support for this interpretation is the fact that inoculations with sap from severely diseased Arran Victory leaves to tomato plants have always transmitted rod-shaped particles, whereas they have sometimes failed with sap from those showing only slight symptoms.

These results give no information about the chemical nature of the rods either in healthy plants or in those infected with paracrinkle virus. The two differ considerably in their resistance to heating; the presumed rods of paracrinkle virus in sap from infected tomato plants began to disappear after heating for 10 min. at 55° C. and none was found after 10 min. at 60°. By contrast, those in healthy Arran Victory, Katahdin and 11–84, all withstood heating to over 90° (Pl. 1, fig. 6). When King Edward sap was heated at 90°, the number of rods was much reduced, suggesting that the paracrinkle virus had been destroyed, and that this variety also contains heat-resistant rods similar to those occurring in other potato varieties. The presumed paracrinkle particles in sap from infected tomato leaves were precipitated by one-third

saturation with ammonium sulphate and redissolved in water apparently unchanged. Few of the rods in sap from uninfected potato plants were precipitated at this salt concentration, but more concentrated preparations will be needed before critical tests on their precipitability can be made.

DISCUSSION

Although our results suggest that paracrinkle virus may be morphologically similar to particles that occur in uninfected potato plants, the two differ so widely in their other properties that there is no reason to assume they are in any way connected. The preliminary studies of the properties of the presumed paracrinkle virus suggest that it resembles such other relatively unstable plant viruses as potato *Y*, which also has rod-shaped particles of about the same width (Bawden & Nixon, unpublished). It is a reasonable assumption that paracrinkle virus may also resemble this in being a protein, but there is no evidence on which to base even a guess at the chemical nature of the rod-shaped particles present in sap from apparently virus-free potatoes. These may also be proteins, but their stability on heating is greater than that of even tobacco mosaic virus, the most heat-resistant of the known rod-shaped viruses, and they could equally well be cellulose. Therefore we have no evidence to suggest that paracrinkle virus is a modification of any constituent of normal potatoes.

Our results do not disprove the contention that paracrinkle virus arose *de novo* in the variety King Edward, though they do seem to make it an unnecessary postulate. The conclusion that the virus is transmitted only by grafting was clearly premature, as also may be the assumption that it occurs naturally only in King Edward. It is readily transmissible to tomato, but this host shows no symptoms and hence infected plants would normally pass unrecognized, and it is extremely unlikely that tomato is the only host that behaves in this manner. The assumption that the virus does not spread in the field and therefore could not have spread to King Edward was based on the fact that there is no record of intolerant potato varieties, such as Arran Victory, being naturally infected. This fact does not prove that infections never occur, but only that they are too few to attract attention. Our results show that Arran Victory, although highly sensitive when infected, is also highly resistant to infection, and the absence of natural infections in it is no evidence that spread may not occur more often to other hosts. There are other viruses that spread rapidly in one host but not in another; for example, dandelion yellow mosaic virus will pass from an infected dandelion to lettuce and then spread rapidly from lettuce to lettuce, but it very rarely spreads from one dandelion plant to another (Kassanis, 1947). Different potato varieties differ widely in the readiness with which they become infected with viruses such as potato *Y* and leaf roll (Bawden & Kassanis, 1946), and this may also be true with paracrinkle virus. As we have no stock of King Edward free from paracrinkle virus, its susceptibility to infection cannot be tested, but it may be higher than that of Arran Victory. If this is so, there is no need to assume that the original seedling contained the virus, for spread from an initial infection occurring later

in the life of the variety could soon have led to the whole clone becoming infected. Also, until King Edward is obtained free from paracrinkle, even to call it a symptomless carrier remains somewhat of an assumption. The variety crops less well than most others that are widely grown, which is one reason for the higher price charged for King Edward tubers, and the leaves are smaller than those of most other varieties and they are also characteristically ruffled and waved. In attempts to obtain virus-free lines for experimental purposes, we have kept King Edward tubers for periods up to 25 days at temperatures around 38°, a treatment effective in curing plants from leaf roll (Kassanis, 1949), but all the tubers that survived the treatment were still infected. As our results from heating *in vitro* indicate a thermal inactivation point of about 55°, this suggests that the virus has a large thermal coefficient of inactivation, a further feature that relates it with other viruses that have rod-shaped particles.

It has long been recognized that effects similar to those caused by viruses are also caused by genes, plasmagenes and hormones, and the relationships between these factors provide a fertile field for speculation. There is nothing intrinsically improbable in the idea that viruses may originate from normal cell components, or that components which are normal in one species may act as viruses when transferred to another, but it cannot be entertained seriously until some evidence is forthcoming that uninfected plants contain particles resembling known viruses. Our results have provided none and have shed no light on the possible origin of paracrinkle virus; they have, however, brought this virus more into line with others that have been studied and thereby abolished the seemingly unique features that have previously been attributed to it. There would seem now to be no need to assume any different relationship between King Edward and paracrinkle virus than that between any other vegetatively propagated host and a virus that it tolerates.

REFERENCES

Barton-Wright, E. C. & McBain, A. (1933). Studies in the physiology of the virus diseases of the potato. II. A comparison of the carbohydrate metabolism of normal with that of crinkle potatoes; together with some observations on carbohydrate metabolism in a 'carrier' variety. *Ann. appl. Biol.* **20**, 525.

Bawden, F. C. (1936). The viruses causing a top necrosis (acronecrosis) of the potato. *Ann. appl. Biol.* **23**, 487.

Bawden, F. C. (1939). *Plant Viruses and Virus Diseases*, 1st ed. Leiden: Chronica Botanica.

Bawden, F. C. & Kassanis, B. (1946). Varietal differences in susceptibility to potato virus *Y*. *Ann. appl. Biol.* **33**, 46.

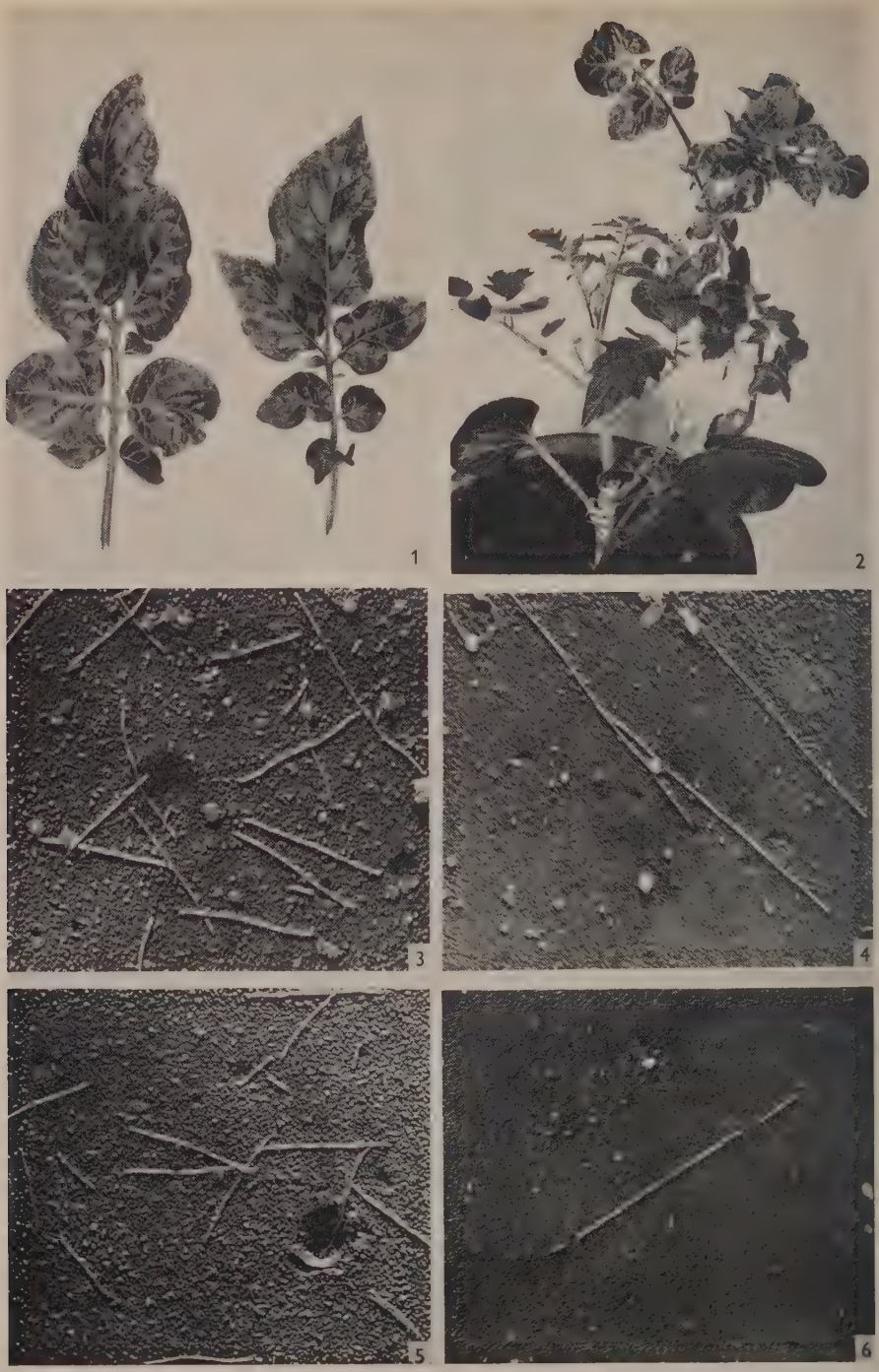
Bawden, F. C. & Roberts, F. M. (1947). The influence of light intensity on the susceptibility of plants to certain viruses. *Ann. appl. Biol.* **34**, 286.

Bawden, F. C. & Roberts, F. M. (1948). Photosynthesis and predisposition of plants to infection with certain viruses. *Ann. appl. Biol.* **35**, 418.

Carson, G. P., Howard, H. W., Markham, R. & Smith, K. M. (1944). Paracrinkle virus and inheritance. *Nature, Lond.*, **154**, 334.

Darlington, C. D. (1944). Heredity, development and infection. *Nature, Lond.*, **154**, 164.

Darlington, C. D. (1949). Les plasmogenes. *Coll. Inter. Centre Nat. Rech. Sci.* **8**, 123.



Figs. 1–6

F. C. Bawden, B. Kassanis & H. L. Nixon—Potato paracrinkle virus. Plate 1

Kalmus, H. & Kassanis, B. (1945). The use of abrasives in the transmission of plant viruses. *Ann. appl. Biol.* **32**, 230.

Kassanis, B. (1947). Studies on dandelion yellow mosaic and other virus diseases of lettuce. *Ann. appl. Biol.* **34**, 412.

Kassanis, B. (1949). Potato tubers freed from leaf-roll virus by heat. *Nature, Lond.*, **163**, 88.

Plank, J. E. van der (1948). Origin of some plant viruses. *Nature, Lond.*, **162**, 291.

Rawlins, T. E. & Tompkins, C. M. (1936). Studies on the effect of carborundum as an abrasive in plant-virus inoculation. *Phytopath.* **26**, 578.

Salaman, R. N. (1932). The analysis and synthesis of some diseases of the mosaic type. The problem of carriers and auto-infection in the potato. *Proc. Roy. Soc.* B, **110**, 186.

Salaman, R. N. & Le Pelley, R. H. (1930). Para-crinkle: a potato disease of the virus group. *Proc. Roy. Soc.* B, **106**, 140.

EXPLANATION OF PLATE

Fig. 1. Leaves from Arran Victory potato plant infected with paracrinkle by inoculation with sap from a diseased Arran Victory.

Fig. 2. Arran Victory potato plant developing paracrinkle after being grafted with a scion from a symptomless tomato plant previously inoculated with sap from a King Edward potato plant.

Fig. 3. Electron micrograph of sap from leaf laminae of an Arran Victory plant with paracrinkle showing rod-shaped particles of variable lengths. ×26,500.

Fig. 4. Electron micrograph of sap from leaf laminae of uninfected Arran Victory plant. The rod-shaped particles are similar to, but fewer than, those in comparable preparations from paracrinkle plants. ×26,500.

Fig. 5. Electron micrograph of sap from leaf laminae of tomato plant inoculated with sap from a King Edward potato plant. No such rod-shaped particles occur in uninfected tomato and they are presumed to be particles of paracrinkle virus. ×26,500.

Fig. 6. Electron micrograph showing rod-shaped particles from uninfected potato plant after heating sap for 10 min. at 98° C. ×26,500.

(*Received* 23 *August* 1949)

An Electron-Microscope Study of Potato Virus *X* in Different States of Aggregation

By A. KLECZKOWSKI and H. L. NIXON
Rothamsted Experimental Station, Harpenden, Hertfordshire

SUMMARY: Electron micrographs of potato virus *X* show tenuous rod-shaped particles of variable length but constant width of about 10 mμ. Lightly shadowed rods lying along the direction of shadowing show a regular cross banding at intervals of about 10 mμ. Attempts to purify the virus cause the particles to aggregate and become entwined to form extensive rope- and net-like structures. This aggregation may be responsible for the loss of solubility that often accompanies purification, though similar structures are visible in micrographs of both soluble and insoluble preparations of the purified virus. Hydrolysis by chymotrypsin yields products too small to be resolvable, and the material remaining after most of a preparation has been hydrolysed appears similar to that in unincubated preparations.

A property that distinguishes potato virus *X* from other plant viruses that have been purified is its tendency to become insoluble when subjected to such procedures as precipitation by acids or salts, ultracentrifugation or dialysis. Insoluble preparations show little or no anisotropy of flow but they are still infective and serologically active; they can be made 'soluble' (i.e. brought into a state in which nothing sediments when liquids are centrifuged at low speeds) by incubation for a short time with trypsin or chymotrypsin, or for a longer time with borate buffer at pH 7·3–7·5 (Bawden & Kleczkowski, 1948). It seemed possible that electron microscopy might reveal some of the causes underlying these phenomena, and for this reason studies were made of the virus in various types of preparation.

MATERIALS AND METHODS

Most work has been done with strain X^y propagated in *Datura stramonium*, a strain-host combination which, of those studied, gives the highest virus concentration in sap. Similar results were obtained with other strains propagated in *D. stramonium* and in other hosts such as tobacco and tomato plants. No differences were observed in the widths of particles of different strains and all showed the same tendency to pass into insoluble forms. Clarified sap formed the starting material; infected leaves were minced in a domestic meat mincer, the juice squeezed through cloth, frozen overnight, thawed, and centrifuged for 10 min. at 8000 r.p.m. The methods of purification used were those of Bawden & Kleczkowski (1948).

Electron microscope mounts were made by placing a droplet containing about 1 part of virus in 10^4 on the usual collodion-covered specimen grid, allowing it to remain in contact for about 1 min. and then sucking away the surplus liquid by bringing an empty micropipette into contact with the liquid surface. Mounts from virus preparations containing added salts were then immediately washed by dipping in distilled water and dried by touching the

edge of the grid on to hard filter paper. Before examination in the R.C.A. model B electron microscope the specimens were shadowed with either 1·5 mμ. of nickel or 1·0 mμ. of palladium, the thickness of the metal film being calculated for the plane of the supporting film, assuming equal spherical distribution about a point source. The shadowing was at an angle of about 12 degrees from the plane of the supporting film.

RESULTS

Size of particles

Pl. 1, fig. 1, shows a typical field in a shadowed preparation made from freshly prepared clarified sap containing virus particles of constant width but greatly differing lengths. The rods are sometimes associated side by side in pairs, but each individual particle is clearly distinguishable. The manner in which they curve suggests a flexibility that contrasts sharply with the apparent rigidity of the wider tobacco mosaic virus particles (Pl. 2, figs. 7, 8). Measurement of particles lying along the direction of shadowing in our sharpest micrographs gives a width of about 10 mμ. for virus *X*, considerably smaller than the 16 mμ. recorded by Takahashi & Rawlins (1946) from measurements on unshadowed micrographs of two strains of the virus. All the strains we have examined had particles of the same width, and none of the treatments we applied affected width, but the apparent width of these very narrow particles does depend upon the sharpness of the image obtained; in Pl. 1, figs. 2, 3, for example, where it is less sharp than in Pl. 1, fig. 1, the particles appear much wider.

In some of our sharpest micrographs, particles that lie along the direction of shadowing show a regular cross-banding at intervals of about 10 mμ. along the length. Further work with enhanced resolution will be needed before this can be interpreted with certainty, but the uniform spacing, coupled with the fact that it is only seen on particles in line with the direction of shadowing, suggest that it reflects a regularity intrinsic to the structure of the particles and is not an artefact caused by uneven deposition or subsequent migration of the shadowing metal.

Effects of ageing and purification

When infective sap is allowed to age, or when the virus is precipitated with acids or salts, the character of the electron micrographs alters strikingly. Instead of separate particles there are elongated masses which are twisted and interwoven to form rope- and net-like structures of various sizes and degrees of complexity. This type of aggregation could well explain the observed decrease in solubility and contrasts sharply with the behaviour of tobacco mosaic virus. Purification of the latter causes the particles to increase in length by joining together end to end, but electron micrographs still show the elongated rods as independent particles. The exact pattern obtained depends upon the manner in which the mount is made; when a drop of purified tobacco mosaic virus suspension is placed on the collodion membrane and allowed to dry, the particles are distributed with their long axes in random directions and largely unassociated (Pl. 2, fig. 7), unless the concentration of the original virus suspension was high enough to give a close-packed or nearly close-packed arrangement.

When the drop is removed after a short interval by sucking with a micropipette, a mount is produced in which most of the particles lie parallel and in contact with each other over considerable areas (Pl. 2, fig. 8). Such particles are never entangled, the orientation being presumably due to flowing liquid during the removal of the drop.

With potato virus *X* the method of preparing the mount for electron microscopy does not affect the manner in which the particles are distributed, similar patterns being obtained whether a drop is allowed to dry undisturbed or is sucked away. The entanglement of virus *X* particles may occur because they are more flexible than tobacco mosaic virus, or because they are more sticky and tend to adhere more readily to each other when they come into contact. The possibility that all these aggregated structures are artefacts produced during the mounting of the specimen cannot be dismissed, but we think it more likely that they reflect differences occurring previously in the virus preparations. The structures do not occur in mounts from freshly extracted material, and they gradually increase in size and complexity as the virus is subjected to an increasing number of treatments before mounting for electron microscopy. Pl. 1, fig. 2, shows a field in a mount made from a virus preparation that had been precipitated once only with ammonium sulphate; most of the particles still remain separate, though a few have become entangled. Pl. 1, fig. 3, is from a soluble virus preparation that was precipitated five times with ammonium sulphate and once with acid. Most of the particles are interlaced to give a typical aggregate. The virus in this preparation became insoluble when dialysed, and Pl. 2, fig. 5, shows the insoluble material. There are no obvious differences from the structures to be seen in Pl. 1, fig. 3, and this we have constantly found. All insoluble virus preparations are aggregated, and electron micrographs do not suggest that they are more so than those preparations which received comparable treatment but remained soluble. Similarly, when soluble preparations are rendered insoluble by the action of ribonuclease (Bawden & Kleczkowski, 1948), micrographs of the precipitated virus are similar to those of the unprecipitated material. So, too, when insoluble preparations are brought into solution by incubation with borate buffer or with trypsin, the micrographs reveal no apparent differences between the state of aggregation before and after treatment. This may mean that the structures are in part produced during the mounting of the specimens, or it may be that solubility is determined by slight differences in the degree of aggregation and that such slight differences are not visible in our electron micrographs.

Effects of trypsin and chymotrypsin

Trypsin and chymotrypsin affect potato virus *X* in several different ways. They hydrolyse the virus, leading to loss of infectivity and all characteristic properties (Bawden & Pirie, 1936), but, depending upon the type of virus preparation used, they may also have other preliminary actions. Preparations consisting mainly of short particles become aggregated (Bawden & Crook, 1947), whereas insoluble preparations can be brought into solution, sometimes within

a few seconds and sometimes more slowly (Bawden & Kleczkowski, 1948). Attempts have been made to follow these changes, but the results are not readily interpretable.

Table 1. *Hydrolysis at 37° of a purified preparation of potato virus X by chymotrypsin*

No.	1·4 % virus in H_2O (ml.)	Buffer* (ml.)	0·4 % chymotrypsin in buffer* (ml.)	Anisotropy of flow (Anis) and serological precipitin titre (Tr) after (hr.) 0 Anis	24 Anis	24 Tr	48 Anis	48 Tr
1	0·5	1·3	0·2	+++	+	1/200	—	1/20
2	0·5	1·5	—	+++	+++	1/2000	+++	1/2000

* 0·2 M borate buffer at pH 7·5.

Table 2. *Hydrolysis at 37° of potato virus X in clarified sap by chymotrypsin*

No.	Sap* (ml.)	Buffer* (ml.)	0·25 % chymotrypsin in buffer* (ml.)	Serological precipitin titre after (hr.) 0	3	6	24
1	1·5	2·25	0·75	1/640	1/320†	1/80†	1/5‡
2	1·5	3·00	—	1/640	1/640	1/320	1/320

* 0·2 M borate buffer at pH 7·5. The pH of the mixtures was slightly under 7·0. It was adjusted to about 7·2 by adding a few drops of 0·1 N-HCl.

† The fluids developed a slight sheen. The appearance of the precipitate was more opaque and granular than in the control.

‡ The fluid contained a small amount of insoluble material which was removed by centrifugation.

Table 1 shows the effects of incubating an aggregated but still soluble preparation of virus *X* with chymotrypsin, the hydrolysis of the virus being estimated by decreases in anisotropy of flow and precipitin titre. At intervals samples were withdrawn for making electron microscope mounts, the dilution being adjusted to keep the concentration of unhydrolysed virus in the mount approximately constant. Pl. 1, fig. 3, shows the material at the start of the experiment; after 48 hr. incubation the unhydrolysed virus, about 1 % of that originally present, still showed the same aggregated structures. Bringing insoluble material into solution by short incubation with chymotrypsin also had no effect on the degree of aggregation as shown in the electron micrographs, and it seems probable that the change from insoluble to soluble state is determined by the size of single aggregates, and can take place by the breaking of large into similar but smaller structures. The hydrolysis of a few cross-linking particles might be enough to do this.

The hydrolysis of individual particles does not seem to occur by their breaking into pieces of successively shorter lengths; instead the whole particle seems to disintegrate into fragments too small to be resolved by the electron microscope.

We have never observed more short particles in an incubated preparation than in unincubated controls, although interpretation is difficult because of the tendency for chymotrypsin to bring about aggregation at the same time as hydrolysis. This effect is shown in Table 2 and Pl. 2, figs. 5, 6. The starting material was clarified infective sap from *D. stramonium* similar to that shown in Pl. 1, fig. 1, in its state of aggregation; after 3 hr. incubation, when about half the virus originally present had been hydrolysed, a sample withdrawn for electron microscopy showed that the remaining virus particles were partially aggregated (Pl. 2, fig. 5). After 24 hr. incubation the preparation contained masses of tightly aggregated material that had become insoluble (Pl. 2, fig. 6). The control, incubated in buffer alone, was not obviously aggregated after 3 hr., but became partially so after 24 hr. incubation.

REFERENCES

Bawden, F. C. & Crook, E. M. (1947). Some properties of potato virus *X* in leaf extracts made in different ways. *Brit. J. exp. Path.* **28**, 403.

Bawden, F. C. & Kleczkowski, A. (1948). Variations in the properties of potato virus *X* and their effects on its interactions with ribonuclease and proteolytic enzymes. *J. gen. Microbiol.* **2**, 173.

Bawden, F. C. & Pirie, N. W. (1936). Experiments on the chemical behaviour of potato virus *X*. *Brit. J. exp. Path.* **17**, 64.

Takahashi, W. N. & Rawlins, T. E. (1946). An electron microscope study of two strains of potato virus *X*. *Amer. J. Bot.* **33**, 740.

EXPLANATION OF PLATES

Plate 1

Fig. 1. Potato virus *X*. Clarified infective sap. The cross-banding is visible on some rods lying along the shadowing direction. Palladium shadowed. ×30,600.

Fig. 2. Potato virus *X*. Precipitated once with 1/3 saturated ammonium sulphate. Nickel shadowed. ×24,700.

Fig. 3. Potato virus *X*. Precipitated five times with 1/3 saturated ammonium sulphate and then once with acid. Soluble. Nickel shadowed. ×28,000.

Fig. 4. Potato virus *X*. The same virus preparation as fig. 3, mounted after dialysis, when it had become insoluble. Nickel shadowed. ×27,300.

Plate 2

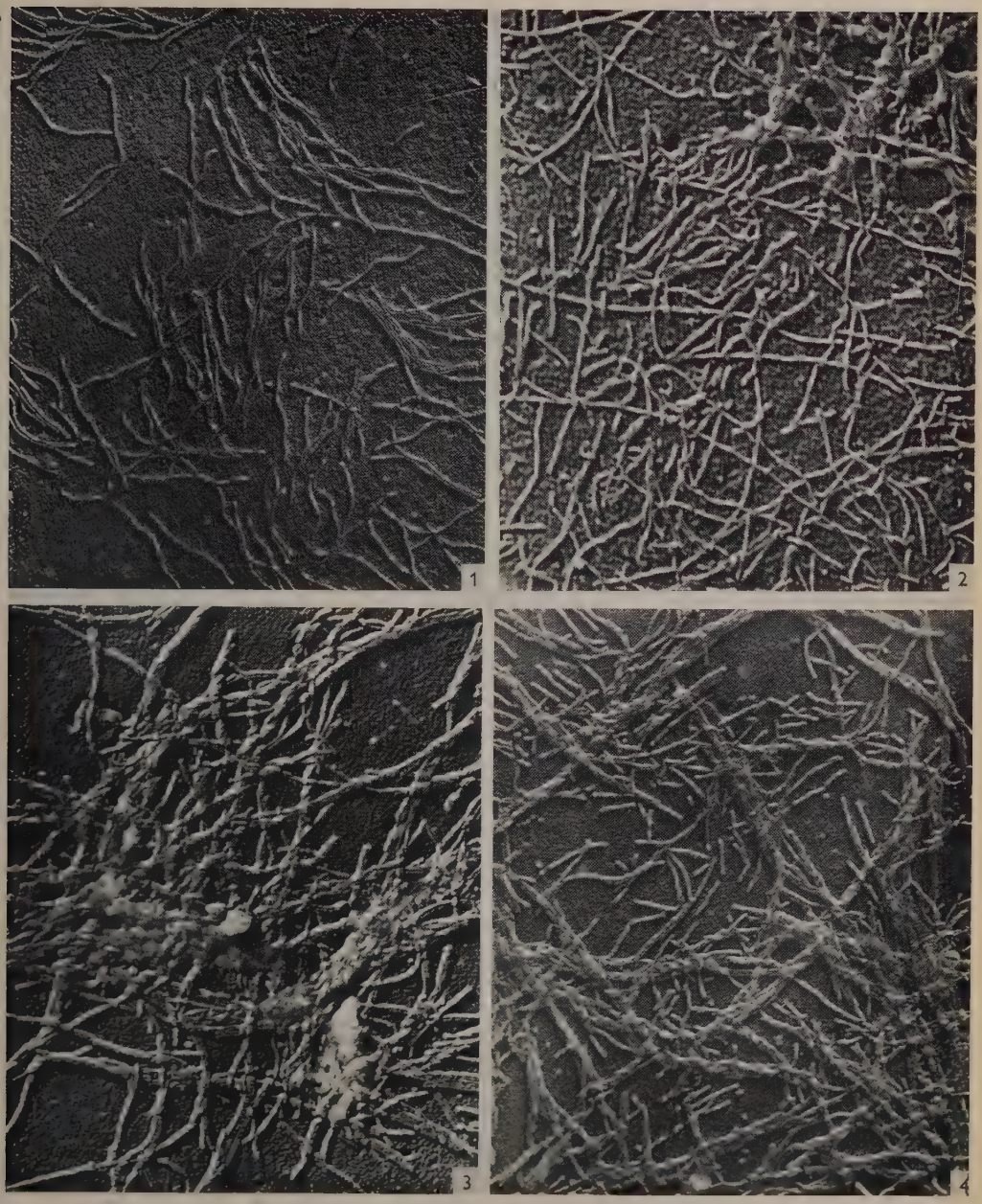
Fig. 5. Potato virus *X*. Clarified infective sap after 3 hr. incubation at 37° C. with chymotrypsin in 0·2M borate buffer at pH 7·2. Palladium shadowed. ×23,400.

Fig. 6. Potato virus *X*. Large aggregates of insoluble material produced by 24 hr. incubation with chymotrypsin as for fig. 5. Palladium shadowed. ×31,400.

Fig. 7. Tobacco mosaic virus. Randomly distributed particles obtained when a droplet of purified virus preparation is allowed to dry on the collodion film. Palladium shadowed. ×36,600.

Fig. 8. Tobacco mosaic virus. Particles oriented when a droplet of the same virus preparation was removed by suction after 1 min. in contact with the collodion film. Palladium shadowed. ×25,300.

(*Received* 23 *August* 1949)



Figs. 1–4

A. Kleczkowski & H. L. Nixon—Aggregation of potato virus *X*. Plate 1

Figs. 5–8

A. Kleczkowski & H. L. Nixon—Aggregation of potato virus *X*. Plate 2

Further Observations on a Filtrable Agent Isolated from Bovine Lumpy Skin Disease

By M. van den ENDE and G. S. TURNER
Department of Pathology, University of Cape Town

SUMMARY: The method of estimating potency of lethal substances by determining death times was applied to the titration of the virus isolated from a case of bovine Lumpy Skin Disease. It was more reliable than that based on the occurrence of recognizable lesions in the embryo after amniotic inoculation.

The method was used to demonstrate the growth of virus in tissues other than skin epithelium, to determine the susceptibility of the virus to destruction by heat, and for the demonstration of neutralizing antibody.

Neutralizing antibody was found in convalescent bovine sera. Normal sera from a variety of animal species including bovines feebly neutralized the virus. This feeble neutralization may indicate the presence in sera of virus-inhibiting substances other than antibody or, in the case of bovines, antibody acquired as a result of inapparent infection.

Van den Ende, Don & Kipps (1949) isolated a filtrable agent from skin and milk-duct nodules from a calf with Lumpy Skin Disease. The agent, which could be transmitted in fertile eggs, passed through collodion filters with average pore diameter of 53 mμ. Its etiological relationship to Lumpy Skin Disease in cattle could not be regarded as proved because the egg-adapted virus after twelve serial passages did not produce Lumpy Skin Disease in cattle, and the results of neutralization tests with sera from normal and convalescent animals were equivocal.

Work with this virus has continued particularly with a view to establishing its etiological role in the natural disease by neutralization tests.

The available titration method was unreliable. It depended on the development of typical lesions in chick embryos surviving for a period of 4 days after inoculation. Many deaths occurred on the second and third day after amniotic inoculation, and these, because they were not associated with recognizable lesions, had to be regarded as non-specific.

Attempts were therefore made to find a more reliable method of titrating the virus and to use it for the detection of neutralizing antibodies. The method finally adopted was also used to measure the adsorption of the virus by chick-embryo tissues, and the resistance of the virus to heat.

METHODS

Material was inoculated into the amniotic cavity, chorio-allantoic cavity or yolk sac of the eggs according to the methods of Beveridge & Burnet (1946). After incubation at 35–36° the eggs were examined by transillumination once or twice a day to ascertain the time of death. In some experiments, sample eggs from each group were opened at different times after inoculation, and the membranes, as well as the embryos, carefully examined for macroscopic lesions.

In a few instances, membranes were examined histologically. In neutralization tests, serum-virus mixtures were held at room temperature for 30 min. and then for 30 min. in a refrigerator, before the eggs were inoculated.

For the production of antiviral serum, adult fowls received repeated intramuscular injections each of approximately one-quarter of a virus-infected chick embryo emulsified in saline. They were bled 10 days after the last of six injections and equal volumes of several sera were pooled.

RESULTS

Yolk and amniotic sac inoculation

As judged by comparative titrations (Table 1) the growth of yolk sac-adapted virus in the yolk sac was as good as the growth of the embryo-adapted virus in the embryo; but there was clearly some yolk sac adaptation because in eggs injected amniotically with embryo-adapted virus the virus content of the yolk sac was less than that in the embryo.

Table 1. *The virus content of yolk sac from eggs injected with yolk sac virus after three yolk-sac passages, and of embryo from eggs infected with embryo-adapted virus*

	Dilution of virus		
	10^{-3}	10^{-5}	10^{-7}
Yolk sac	2 typical lesions, 4 dead	5 typical lesions, 1 dead	6 normal
Embryo	3 typical lesions, 3 dead	3 typical, 3 doubtful early lesions	5 normal, 1 doubtful lesion

Allantoic cavity inoculation

Attempts were made to adapt the virus to growth in the chorio-allantois rather than the amnion. Embryonic fluid from eggs inoculated by the open amniotic route was collected; when diluted 1/100 in normal saline, 0·2 ml. almost invariably killed all the embryos in 2–4 days. At first this high mortality was thought to be due to unrecognized contaminants, but it occurred regularly in spite of careful elimination of contamination and the subsequently proved absence of demonstrable bacteria.

Fluid from an embryo infected by the allantoic route, which had survived to the third day, was injected allantoically into groups of eggs, diluted in normal saline 10^{-1}–10^{-6}. The membranes of dead eggs were examined daily, when necessary, by teasing out in saline and inspecting by darkground illumination with a hand lens. Lesions appeared inconstantly, and none was striking. Many of the membranes were thickened and in parts gelatinous. Usually there was a widespread greyish opacity, particularly in the vicinity of the blood vessels, and some membranes had innumerable tiny focal opacities. Histologically the opacities were usually composed of amorphous material which dissolved during the preparation of the section. Only occasional focal areas of cellular proliferation or infiltration were seen.

The mortality record (Table 2) revealed that virus multiplying in the chorio-

Table 2. *The average day of death* (A.D.D.) *of eggs after inoculation of virus into the allantoic cavity*

Dilution of fluid	No. dying on (day) 1	2	3	4	5	6	7	No. surviving on 7th day	A.D.D.
10^{-1}	1*	1	2	6	—	—	—	1	3·3
10^{-2}	—	—	2	6	1	—	—	—	3·9
10^{-3}	—	1	1	3	2	1	2	—	4·7
10^{-4}	—	—	—	3	5	2	—	—	4·9
10^{-5}	—	—	—	2	5	3	—	—	5·1
10^{-6}	—	—	—	1	1	1	—	7	—

* Death regarded as non-specific and not included in calculation of A.D.D.

allantoic membranes kills with greater regularity than virus injected into the amniotic cavity, and that the time of survival of embryos increased with increasing dilution of inoculum.

Virus reached a higher titre in embryonic fluids after amniotic than after allantoic inoculation (Table 3). This difference persisted, though to a lesser degree, after continued serial allantoic passage.

Table 3. *Comparative titrations of virus in combined amniotic and allantoic fluid of eggs inoculated either into the amniotic or allantoic space*

Fluid from egg inoculated into the amniotic sac

Dilution of fluid	No. dying on (day) 1	2	3	4	5	6	7	8	No. surviving on 10th day	A.D.D.
10^{-3}	—	1	3	8	7	1	—	—	—	4·2
10^{-4}	1*	1	1	8	7	2	—	—	—	4·4
10^{-5}	1*	1	—	2	6	2	1	3	4	5·6
10^{-6}	4*	—	—	2	—	1	1	—	12	—
10^{-7}	—	1	—	2	—	—	—	—	17	—

Fluid from egg inoculated into chorio-allantoic sac

Dilution of fluid	No dying on (day) 2	4	5	6	7	8	9	No. surviving on 10th day	A.D.D.
10^{-2}	—	4	10	4	1	1	—	—	4·7
10^{-3}	—	1	7	6	1	2	1	2	5·9
10^{-4}	—	—	1	3	—	—	—	16	—
10^{-5}	—	3	—	—	—	—	—	17	—
10^{-6}	1	—	—	2	—	—	—	17	—

* Not included in calculation of A.D.D.

From the results in Table 3 the LD50 of virus (Reed & Muench, 1938) in the two groups was $10^{-5\cdot3}$ and $10^{-3\cdot6}$ respectively. Furthermore, there was here also a progressive increase in survival time of eggs with increasing dilution of inoculum. This was suggestive of the results obtained by Golub (1948) for

the titrations of psittacosis virus. A larger titration (Table 4) confirmed the relationship between average survival time and virus inoculum.

Table 4. *Titration of virus in allantoic cavity*

Dilution of fluid	No. dying on (day) 1	2	3	4	5	6	7	8	9	No. surviving on 10th day	A.D.D.
10^{-0}	—	—	6	8	1	—	—	—	—	—	3·7
10^{-1}	—	—	2	3	6	4	—	—	—	—	4·8
10^{-2}	—	—	2	3	—	2	1	4	—	3	6·1
10^{-3}	—	1	—	1	4	—	1	2	—	6	6·3
10^{-4}	1*	—	—	—	1	2	1	—	2	8	—

* Not included in calculation of A.D.D.

The results recorded in Tables 3 and 4 have also been recorded graphically (Fig. 1) according to the method described by Golub. Although the values for the average day of death with different inocula are roughly linear with respect to dose in any one titration, the slopes vary with titrations, so that the average day of death after inoculation of a single, certainly fatal dose of virus could not be regarded as an accurate estimate of virus content in different fluids.

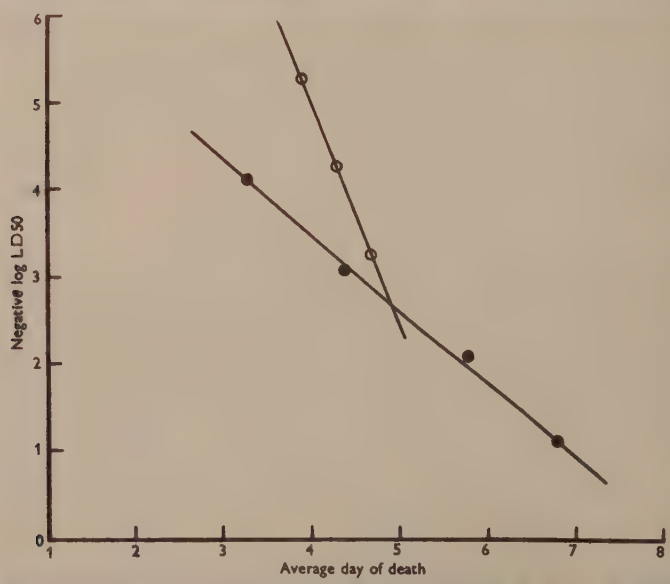


Fig. 1. Relationship between dose and death time (average day of death) of eggs receiving virus allantoically. ○——○, embryonic fluids, LD50 $10^{-4\cdot13}$ (groups of fifteen eggs); ●——●, embryonic fluids, LD50 $10^{-5\cdot3}$ (groups of twenty eggs).

In all subsequent experiments virus was titrated by estimating average death times of embryos inoculated by the allantoic route. The method proved to be convenient for comparing the neutralizing potency of antisera in any one test.

Demonstration of neutralizing antibody

In antisera. Groups of eggs received the same dose of virus mixed (*a*) with broth, (*b*) with serum from fowls immunized by repeated intramuscular injections of the virus, and (*c*) with serum from normal fowls.

There was (Table 5) a marked decrease in the mortality rate with fowl anti-serum, whereas serum from normal fowls only slightly increased the average survival time.

Table 5. *Showing the virus-neutralizing power of immune fowl serum*

		No. dying on (day)							No. surviving on 10th day	A.D.D.
		1	2	3	4	5	6	7		
	Broth	1*	1	1	8	7	2	—	—	4·3
Virus 10^{-4}	Normal fowl serum	1*	—	3	7	4	2	1	2	4·5
	Immune fowl serum A	—	—	—	—	1	1	1	17	—
	Broth	—	1	7	6	1	—	—	—	3·5
Virus 10^{-3}	Normal fowl serum	—	—	2	9	4	—	—	—	4·1
	Immune fowl serum B	—	—	—	—	—	1	—	14	—

* Not included in calculation of A.D.D.

In convalescent bovine sera. Table 6 shows typical results with sera supplied by The Onderstepoort Veterinary Laboratories. The sera were also tested by the orthodox method based on the inhibition of the development of lesions after amniotic inoculation. Because the number of eggs used was small the results were not analysed statistically. The neutralizing power of all the sera was relatively poor. The death-time method was no less sensitive than the other. The results illustrate the relative simplicity and ease of interpretation of the former test, the latter being complicated by the occurrence of doubtful lesions and deaths, which because of the absence of typical lesions had to be regarded as non-specific. The slight neutralization was evident both from the decrease in mortality and from the increase of average survival time, even after inoculation of serum-virus mixtures that killed all the eggs. However, with small doses of virus, interpretation was difficult owing to the slight non-specific neutralization by normal serum.

A statistical analysis was profitable only with the results with relatively large groups of eggs. These experiments (Table 7) were made with fowl anti-serum of high potency, and the purified globulin fraction from colostrum of a cow which had had Lumpy Skin Disease. The results show that dilutions of serum

Table 6. *Neutralization tests using the allantoic and amniotic routes of inoculation*

Allantoic route

Serum	Dilution of virus 10^{-3} No. dying on (day) 2	3	4	5	6	7	Survived	10^{-4} No. dying on (day) 2	3	4	5	6	7	Survived	10^{-5} No. dying on (day) 2	3	4	5	6	7	Survived	Neutralization
Immunized bovine	—	—	—	1	—	1	4	—	—	—	—	1	1	4	—	—	—	—	—	—	6	+
Normal bovine	—	1	2	2	—	—	1	—	—	1	2	—	1	3	—	—	1	—	—	—	5	±
Nil	—	2	3	1	—	—	—	1	—	3	—	2	—	—	—	1	3	2	—	—	—	—

Amniotic route

Serum	Dilution of virus 10^{-6}	10^{-8}	10^{-10}	Neutralization
Convalescent bovine	4 normal, 3 dead	4 normal, 3 dead	3 normal, 4 dead	+
Normal bovine	4 typical, 1 doubtful lesions, 1 dead, 1 normal	3 typical, 1 doubtful lesions, 1 dead, 2 normal	1 typical lesions, 5 dead, 1 normal	—
Nil	3 typical lesions, 4 dead	4 typical lesions, 2 dead, 1 normal	1 typical lesions, 6 dead	—

Table 7. *Neutralization tests (allantoic inoculation), using immune fowl serum and colostrum globulin from convalescent bovine*

Amniotic route

	No. dying on (day)														No. surviving on 10th day	A.D.D.
	1½	2	2½	3	3½	4	4½	5	5½	6	6½	7	7½	8½		
Immune fowl serum																
1/20	—	—	—	—	1	—	2	—	1	1	—	—	—	—	7	—
1/40	—	—	—	—	3	—	3	—	3	—	1	—	—	1	1	5·0 ± 1·5
1/80	—	—	—	—	1	1	2	1	2	—	1	1	2	—	1	5·5 ± 1·3
1/160	—	1	—	1	3	—	4	—	2	—	—	—	—	—	1	4·0 ± 1·0
1/320	1	—	—	2	2	1	5	1	—	—	—	—	—	—	—	3·8 ± 1·0
Broth control	1	1	5	5	—	—	—	—	—	—	—	—	—	—	—	2·6 ± 0·5
Colostrum																
1/8	—	1	—	—	—	2	—	—	—	1	—	—	—	—	8	—
1/16	—	1	—	2	—	3	—	2	—	—	—	—	—	—	4	3·7 ± 1·0
1/32	—	—	—	—	—	6	—	5	—	1	—	—	—	—	—	4·6 ± 0·7
1/64	—	1	—	—	—	7	—	3	—	1	—	—	—	—	—	4·2 ± 1·0
Broth control	—	1	—	5	—	6	—	—	—	—	—	—	—	—	—	3·4 ± 0·7

or colostrum insufficient to prevent death of any of the eggs nevertheless increased the average survival time.

It therefore appears to be sufficient in order to demonstrate neutralizing antibody qualitatively to use only one group of eggs for each serum under test. Even a ten-fold reduction in the amount of virus injected, however, increases the average survival time only very little, so that the neutralization test based on an increase in average survival time can only be reliable if large groups of eggs are used and if due allowance is made for the fact that some apparent neutralization is shown by many normal sera from a variety of animal species (Table 8).

Table 8. *The neutralizing power of normal sera*

	Average day of death or proportion of survivors	
	Virus 10^{-3}	Virus 10^{-5}
Broth control	4·2	5·1
Human serum	4/10	4/11
Fowl serum	3·5	2/11
Guinea-pig serum	8·3	7/11

Surviving eggs were discarded after 12 days.

A large number of bovine sera were available for test, but most of them were obtained long after the animals had recovered from the disease. Furthermore, apparently normal bovines may have had subclinical infection and these sera consequently were not 'normal'. So far, striking neutralization was observed only with serum from animals that had recently recovered from the disease or had been used recently in transmission experiments. On the other hand, sera from convalescent animals were encountered in which demonstrable antibodies were scanty or absent.

Absorption experiments

The surface adsorption of a number of viruses by red blood corpuscles or tissue cells encouraged us to attempt the demonstration of similar adsorption of this virus by susceptible cells. The failure of virus suspensions to agglutinate red blood cells from a variety of animal species has already been reported (van den Ende *et al.* 1949).

Several attempts were made with emulsified skin from 9-day chick embryos. Skin was chosen because bovine virus is localized in the deeper layers of the skin and because the lesions in the chick embryo appeared to occur mainly in the skin or its appendages.

Skin was peeled off twelve 9-day-old chick embryos, emulsified in 10 ml. normal chick allantoic fluid containing 50 u. of penicillin and 50 μg. of streptomycin. The centrifuged deposit from this emulsion was thoroughly mixed with 10 ml. of a 1/20 dilution in normal fluid of allantoic fluid from a virus-infected egg, held at room temperature for 30 min. and centrifuged. The supernatant fluid, the deposit ground up in 10 ml. normal allantoic fluid, the original virus suspension (1/20), and a control virus suspension subjected to the same centri-

fugation as the virus-skin mixture were each tested for virus content. A single group of 10 eggs was used for each titration. The average day of death in these titrations was 4·5, 5·3, 4·4 and 4·7 respectively. There was, therefore, no demonstrable adsorption by skin tissue under the conditions of the experiment.

Heat stability

The method was also used to measure the heat stability of the virus (Table 9). The virus is completely destroyed at 80° in 5 min. Some of the virus survives 60° even for 1 hr., but even 15 min. at this temperature is sufficient to inactivate some of the virus.

Table 9. *The susceptibility of virus to heat*

Heating		No. dying on (day)						No. surviving on 10th day	A.D.D.
Temp. (° C.)	Time (min.)	2	3	4	5	6	7		
100	5	—	—	—	—	—	—	10	—
80	5	—	—	—	—	—	—	10	—
60	60	—	—	—	2	1	2	5	—
60	30	—	—	2	1	4	—	3	—
60	15	—	3	5	1	1	—	—	4·0 ± 0·6
Nil	—	1	9	—	—	—	—	—	2·9 ± 0·2

DISCUSSION

It was clear from the outset that the proof whether or not this virus was the cause of Lumpy Skin Disease would be difficult. If the virus is the cause of the disease it must be assumed from experiments carried out on bovines that it has lost its ability to elicit the characteristic disease in its original host. Such attentuation of animal viruses by passage through eggs is well known. Its identification must then depend on the determination of its physical and chemical properties as well as its antigenic structure.

A serious handicap in the study of this virus was the difficulty of exact titrations. The new method outlined, although itself imperfect, is simple and gives results that can be subjected to statistical analysis. A limited supply of eggs precluded the confirmation of the results recorded above, by tests designed to give statistically significant results, but it is hoped that the method will be studied in laboratories where liberal supplies of fertile eggs are available.

But even in a limited application the method proved useful in demonstrating growth in various tissues of the egg other than the skin epithelium, for which the virus at first appeared to have a special affinity. The absence of a special affinity for skin epithelium is further shown by the non-adsorption of the virus by embryo skin *in vitro*.

The virus can be easily adapted to growth in the allantoic cavity, causing death in 3–8 days, but without the constant production of recognizable lesions. Virus also multiplies in the yolk sac and in eggs infected by this route the characteristic skin manifestations sometimes develop in the embryo. The virus therefore appears to become rapidly generalized throughout the tissues of the egg. On the other hand, only direct inoculation into the amniotic cavity will

regularly cause the characteristic skin and feather follicle changes previously described.

In neutralization tests sufficiently striking results have so far only been obtained with serum from immunized fowls, a sample of globulin from bovine colostrum and the occasional serum from convalescent bovines. Neutralizing antibodies in the serum of most convalescent bovines were present in relatively small amounts only.

Neutralization by the serum of apparently normal bovines at first sight might suggest that the virus has no relation to Lumpy Skin Disease, but alternative explanation is possible in the light of the known epidemiology of Lumpy Skin Disease and its behaviour in experimental animals. It is well known, for instance, that in many herds the natural disease is clinically recognizable in only a small proportion of animals. It is not unlikely that the remaining animals had experienced mild subclinical infections. This would explain the sudden disappearance of the disease from most parts of the country, and could well account for the difficulty of experimental transmission of the disease even to apparently normal animals. On the other hand, neutralization by normal serum not only from bovines, but also from man, guinea-pigs, and fowl may be the property of substances other than antibodies, and analogous to the influenza virus inhibiting substances normally encountered in serum. Substantiation of some of these arguments and proof of the identity of the virus was obtained by Dr R. Alexander at Onderstepoort who, in a personal communication, reports that virus after sixty-seven serial egg passages was given subcutaneously to four normal bovines bred in quarantine. Two of these developed typical generalized Lumpy Skin Disease. Why the original attempts to infect cattle with egg virus failed cannot be readily explained. The cattle used in the first attempt may have been immune in spite of not having shown clinical manifestations of previous infection. This is supported by the presence of neutralizing antibodies in the serum of apparently normal cattle. Furthermore, the virus used in the first bovine experiments was of lower titre and had been transported on its 1000-mile air journey in dry ice in which it slowly froze.

We are grateful to Mr J. H. Maytham for his assistance in carrying out this work; Dr R. Alexander and Dr A. Polson of Onderstepoort very kindly gave the colostrum globulin, many serum samples and invaluable advice.

This investigation was assisted by a financial grant from the Council for Scientific and Industrial Research for the purchase of fertile eggs.

REFERENCES

Beveridge, W. I. B. & Burnet, F. M. (1946). The cultivation of viruses and rickettsiae in the chick embryo. *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 256.

Ende, M. van den, Don, P. A. & Kipps, A. (1949). The isolation in eggs of a new filtrable agent which may be the cause of bovine Lumpy Skin Disease. *J. gen. Microbiol.* 3, 174.

Golub, O. J. (1948). A single-dilution method for the estimation of L.D. 50 titres of the Psittacosis-L.G.V. group of viruses in chick embryos. *J. Immunol.* 59, 71.

Reed, L. J. & Muench, H. (1938). A simple method of estimating fifty per cent endpoints. *Amer. J. Hyg.* 27, 493.

(*Received* 25 *August* 1949)

An Improved Method for the Preparation of Silica Gel Media for Microbiological Purposes

By C. B. TAYLOR

I.C.I. Ltd., Butterwick Research Laboratories, The Frythe, Welwyn, Hertfordshire

SUMMARY: A base exchange column for the preparation of silica sols is described. Sols prepared by the method can be sterilized by heat treatment and are stable for some weeks. Bacteriological media can easily be prepared with such sols and the gelling time regulated by the concentration of sodium chloride used.

For many microbiological purposes it is either essential or preferable to use media solidified by an inorganic gel; among inorganic gels only silica gels are at present practicable. The various published methods of preparing silica gels are far from satisfactory. Silica gel media are usually prepared by neutralizing sodium silicate with hydrochloric acid. In one modification the gel is allowed to set in a Petri dish and then washed for 24 hr. under running water and finally sterilized in a hot-air oven; the applications of this method are very restricted, and there is no guarantee that toxic substances are removed in the washing process. In another method the silicate-acid mixture is dialysed for several days through a membrane, and the purified product sterilized by filtration, gelation taking place rapidly after this operation; though the resulting gel is excellent the method is very time-consuming and tedious and the speed of gelation cannot be controlled.

A greatly improved method is possible by making the gel from a silica sol, removing the cations from sodium silicate by means of base-exchange material. To Joseph Crosfield and Sons Limited, I am greatly indebted for a supply of materials and for technical information which, with only minor modifications, is the basis of the method for preparing the sol described below. Media prepared with the silica sol supported excellent growth of all bacterial strains tested and a description of the methods may be of value to workers in various fields of microbiology.

PRODUCTION OF THE SILICA SOL

The base-exchange column

A glass tube of about 2 in. in diameter and 3 ft. long is held vertically by two large clamps (Pl. 1). At the base of the tube a plug of glass-wool is supported by short pieces of glass rod which rest on a tightly fitting rubber bung. A glass tube having a side arm and, below, a stop-cock is inserted up through the bung so that the top of the tube is level with the top of the bung. The tube is charged with 1 l. (approximately $1\frac{3}{4}$ lb.) of 'Soucol', the base-exchanging material, leaving 10–12 in. of free space at the head of the column.

The bed of Soucol is graded by back-flushing with tap water through the side arm. This operation is repeated several times, draining the water till level with the top of the column of Soucol ('bed level') on each occasion. If the column

separates during the back-flushing process the upper portion can be brought down most satisfactorily by a gentle rotational movement produced by rocking the tube. The column should finally be quite free from air bubbles. The bed is then washed with 2 l. of distilled water, and having made certain that the flow from the discharge tube is not restricted, the liquid is drained to bed level.

The Soucol is regenerated by placing 300 ml. of 10 % (v/v) hydrochloric acid in the free portion above the bed and allowing it to flow at *c.* 50 ml./min. When bed level is reached a further 200 ml. of the acid is passed through. Finally the column is washed with 2 l. or more of distilled water until the effluent is free of chlorides, and drained to bed level. If at any time either water or acid drains below bed level it is necessary to back-flush to remove air bubbles and repeat operations.

The concentration of sodium silicate used depends on the purpose for which the product is required, but a 3 % SiO_2 sol is the most satisfactory for general purposes. Concentrations much greater than 3 % SiO_2 should be avoided because the bed may become gelled.

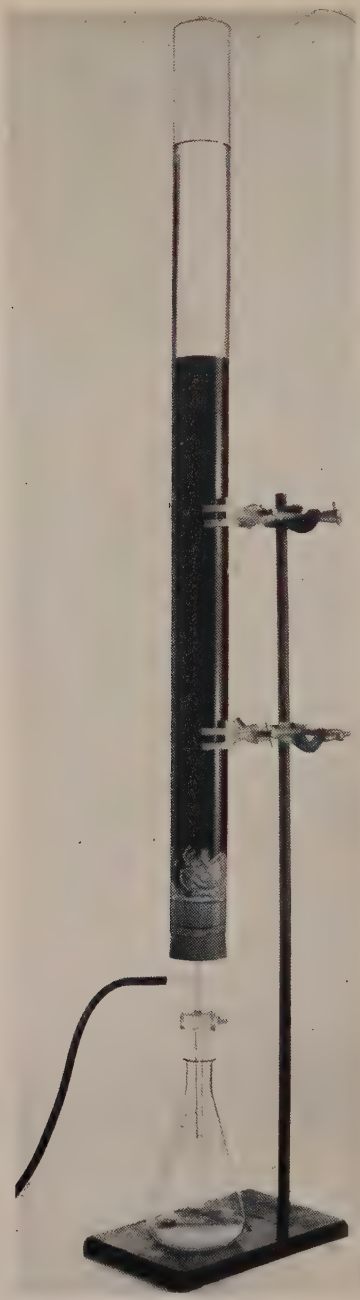
Neutral sodium silicate 78/80 degrees Twaddel containing 28·9 % SiO_2 (Crosfield and Sons Ltd.) is diluted to a concentration of 312 g./3 l. of distilled water, and the solution passed through the bed at a speed of 100 ml./min. The first 500 ml. of the effluent, consisting largely of water, is discarded. The bed is then completely drained. It is advisable to test the pH of the effluent from time to time to ensure that it is acid. If it becomes alkaline the base exchange capacity of the bed is exhausted, perhaps because the Soucol was not properly regenerated. As the addition of cations will affect the gelling properties of the sol it is better to discard any alkaline sol or sols contaminated with such cations. The bed is then back-flushed and all air bubbles removed, washed down with 2 l. of distilled water, drained to bed level and regenerated. The sol produced should be perfectly clear and almost colourless.

An analysis of a prepared sol showed the following substances, expressed as p.p.m.: organic matter (as C), 185; Ca, 15; Mg, 10; Pb, 8; Zn, 4; Cu, <1; P_2O_5, 2.

Preparation of media

The only difficulty in using the silica sol in bacteriological media is the estimation of the time required to gel. As gelling depends, among other things, upon the strength of the sol, temperature, and the nature and concentration of the substance to be added, it is not possible to state precisely the method of preparing any particular medium. Small trial batches should be made until the correct conditions have been determined for setting the gel within the required period.

The sol, which has an initial pH value of *c.* 3·0, is stable over a period of at least 3 weeks, and becomes increasingly opaque with storage. It can be sterilized at 15 lb. pressure in an autoclave without affecting its gelling properties, but this operation decreases the acidity appreciably. The sol cannot, however, be adjusted to pH 7·0 and subsequently sterilized, because this procedure destroys the gelling power. Moreover, if the sol has not been properly freed from cations it will gel during autoclaving. Although gelling can be obtained by adding the



C. B. Taylor—Silica gel for media. Plate 1

usual salts present in peptones and meat extracts, the process is slow, and it is advisable to control gelling time by suitable concentrations of sodium chloride. If desirable, an ammonium gel can be formed by the addition of ammonium compounds, such as $(NH_4)_2SO_4$. When rapid gelling is not required, as in the preparation of slopes, stab cultures, or for the inoculation of the surface of media in Petri plates the addition of 0·1 % NaCl will allow peptone-yeast extract medium to gel in approximately 2 hr. when the SiO_2 content is 1·5 %. When plate counts are to be carried out and gelling is required within 10–20 min. the concentration of NaCl required may be as great as 0·5 %, but this will depend upon the nature and concentration of the ingredients; a simple salt medium containing appreciable amounts of certain salts, particularly ammonium salts, will gel rapidly.

It is essential to sterilize the silica sol separately from the ingredients of the medium. To prepare a litre of any medium the total quantities of salts and other ingredients are dissolved in 250 ml. of distilled water and adjusted to the required pH value. The amount of N-NaOH required to adjust 750 ml. of the *sterilized* sol is determined, and this amount added as excess to the 250 ml. of solution containing the ingredients. The two sterilized solutions are mixed when required, and the necessary amounts pipetted into plugged sterilized tubes or into Petri dishes. When colony counts are to be made, smaller quantities are mixed, sufficient for 3–4 plates. Very satisfactory results have been obtained by preparing 15 ml. of sol and 5 ml. of nutrients separately in test tubes. The measured amount of fluid is placed in the Petri dish and mixed with the sol. The nutrients are then added, and after thorough mixing, the dish is allowed to stand for 1 hr. before inverting. Properly prepared plates require no drying and can be inverted in the incubator in the usual manner. For counts in milk, soil or polluted water, it should be possible to incorporate the NaCl in the dilution fluid and thus eliminate the danger of the medium gelling before use. For semi-solid media a final SiO_2 content of 0·5–0·75 is satisfactory; for slopes the concentration should be at least 2·5 %.

The easy manner in which silica sol can be produced, its stability during storage and autoclaving suggest that, with ingenuity, the microbiologist can use silica sol for many purposes in the cultivation of micro-organisms. My occasional failures in preparation have invariably been due to the production of an incorrect sol, either by the use of a sodium silicate other than the brand stated above, or of an exhausted bed of Soucol.

It should be noted that a patent has been filed in Britain for the production of silica sols by passage through carbonaceous base-exchange material by the National Aluminate Corporation of America.

EXPLANATION OF PLATE

Base-exchange column for preparation of silica sols.

(*Received* 9 *September* 1949)

The Production of γ-Aminobutyric Acid by *Bacterium coli* Wilson, Type I

By H. K. KING and L. I. FLETCHER
The Bacteriology Department, University of Edinburgh

SUMMARY: Examination by paper partition chromatography of casein-hydrolysate/glucose culture filtrates of forty-seven strains of coliform organisms indicates that the decarboxylation of glutamic acid is characteristic of Wilson's *Bacterium coli* Type I.

Linggood & Woiwod (1948) have drawn the attention of bacteriologists to the potentialities of paper partition chromatography as a means of investigating the nitrogen metabolism of bacteria. The amino-acids present in a single drop of a culture filtrate may be recognized by this simple and elegant technique, and a large number of samples investigated simultaneously. During a study of amino-acid utilization by a variety of bacteria it was observed that certain members of the *Bacterium coli* group readily utilized glutamic acid with production of a substance whose chromatographic behaviour led to its identification as γ-aminobutyric acid. Gale (1940) has shown that some strains of *Bact. coli* possess a glutamic decarboxylase enzyme and demonstrated its action in washed suspensions. Through the courtesy of Dr J. P. Duguid and Miss M. J. D. Macpherson, of this Department, we obtained a number of strains of coliform organisms of known taxonomic characteristics. We found that the production, on a casein-hydrolysate glucose medium, of what is most probably γ-aminobutyric acid, is characteristic of Wilson's (1935) '*Bact. coli* Type I'.

EXPERIMENTAL

Bacteria. These are listed in Table 1. Most of them were recently isolated by Miss M. J. D. Macpherson from the sources indicated, or obtained from the National Collection of Type Cultures without information as to their origin. The classification of Wilson (1935) has been followed where it is applicable.

Media. Casein was hydrolysed with 6 N hydrochloric acid for 45 min. at 120°, and the bulk of the acid removed by repeated vacuum distillation. Phosphates were removed by precipitation at pH 9. The medium was finally diluted to contain the equivalent of 2 % casein, the pH adjusted to pH 7·4, and 0·5 % of glucose added. Sterilization was by a single steaming for 1½ hr., sterility being tested by incubation before use. Enrichment with growth factors (cf. Proom & Woiwod, 1949) was not necessary, since we were investigating only Gram-negative organisms with relatively simple nutritional needs.

The sodium chloride present in this medium produced a yellow spot (R_F 0·20) on the collidine chromatograms. This salt can be removed if desired by careful alternate treatment with cation- and anion-exchange resins; the medium should then be re-examined chromatographically to ensure that no amino-acids have been lost.

Procedure. A tube of the casein-hydrolysate glucose medium was inoculated

with the appropriate strain from an agar slope culture and incubated for 72 hr. at 37°. The bacteria were removed by centrifugation and the supernatant fluid examined chromatographically; the pH of the fluid was noted.

Chromatographic methods. A general account of the technique has been given by Consden, Gordon & Martin (1944), Dent (1948), and Woiwod (1949), so no detailed description is needed. Single-dimensional chromatograms were run on

Table 1. *Strains of bacteria used*

Classification	Reactions: Methyl red	Vosges Proskauer	Elkjman	Indole	Citrate	Origin: Faeces	Wounds	Urine	Water	Milk	Laboratory Stock	N.C.T.C.
Bact. coli Type I	+	−	+	+	−	E48–9, 55, 93, 97–8, 101–4, 106	E50, 51, 52	E57, 58, 92	E53, 54	.	E56	123, 414, 419, 5928, 7275
Bact. coli Type II	+	−	−	−	−	E91, 95, 96	.	.	.	.	.	.
Intermediate I	+	−	−	−	+	.	.	.	.	.	.	3735
Bact. aerogenes Type I	−	+	−	−	+	.	E59	.	.	.	E1, 10, 14, 29, 61	418
Bact. aerogenes Type II	−	+	−	+	+	.	.	.	.	.	.	5936
Bact. anaerogenes	+	−	−	+	−	.	.	.	E119	E121	.	.
Bact. cloacae	−	+	−	−	+	.	.	.	.	.	.	5920
Irregular	−	−	−	−	+		.	.	.	.	.	7271
	+	−	+	+	+	E60 .	.	.	.	.	.	.
Bact. pneumoniae	+	−	−	−	−	.	.	.	.	.	E30	
	+	+	−	−	+	.	.	.	.	.	.	5054
	−	+	−	−	+	.	.	.	.	.	.	5055
	+	−	−	−	+	.	.	.	.	.	.	5056
Bact. ozaenae	+	−	−	−	+	.	.	.	.	.	.	5050

strips of Whatman No. 1 filter paper, 8 × 16 in., suspended from troughs 9 in. long made from Pyrex tubing (Atkinson, 1948). These glass troughs were strong, easily made and avoided the expense of stainless steel or the difficulties arising from the use of less resistant metals. The samples (0·01 ml.) were measured from an 0·1 ml. blood pipette on to the paper strip, the end of which was folded round a glass strip which was then lowered into the trough. Five samples were run on each strip, with a control sample of the uninoculated medium at each end. The troughs were held in carriers cut from tinplate, care being taken that the papers did not come into contact with the metal. The carriers were suspended in glass pathological specimen tanks, 18 × 16 × 15 in., covered with a sheet of glass or asbestos. This simple improvised apparatus proved wholly satisfactory.

Each culture-supernatant was examined in two solvent systems: (1) phenol-water with 0·1 % ammonia (redistilled phenol being used); (2) collidine-water, the commercial collidine, containing a mixture of isomers, being redistilled once before use.

The solvent phase was placed in the trough, with a beaker containing both phases in the bottom of the tank. Phenol chromatograms were run until the

solvent-front had nearly reached the end of the paper strip (about 24 hr.). The collidine chromatograms were run for 4 days, i.e. the solvent-front was allowed to run right off the strip. This was desirable since none of the amino-acids have R_F values greater than 0·60 and most are crowded into the region above R_F 0·35.

The papers, after removal from the troughs, were dried by suspending over a radiator or electric hot-plate, no special drying chamber being available. The solvent fumes would, however, cause annoyance if many papers were treated thus. The dry papers were sprayed with 0·1 % ninhydrin in *n*-butanol, allowed to dry at room temperature, and then placed in the 37° incubator for 3 hr. to develop the spots. Like Dent (1948) we found that heating in an oven as recommended by the original authors (Consden *et al.* 1944) was liable to cause rapid fading. Our chromatograms were stable for about a month, and were photographed when permanent records were required.

No constant-temperature room was available, but an inner room without windows was found to keep within 1° over a 24 hr. period and seldom varied by more than 2° even during the 4-day runs with the collidine system. The actual temperature is less important than its constancy during a run, though collidine does not give satisfactory results above about 18°, and temperature must always be taken into account in interpreting collidine chromatograms.

RESULTS

Decarboxylation of glutamic acid. With certain strains the glutamic acid spot was absent from the chromatograms of the culture filtrate and a new spot was seen. Experiments with shorter incubation periods showed that these changes occurred concurrently. The R_F value of the new spot was 0·80 in phenol—i.e. it appeared as a strengthening of the valine spot; in collidine the R_F value was 0·20 and its position immediately below that of glutamic acid itself. The production from glutamic acid of material giving the new spot was confirmed when one of the strains concerned, *Bact. coli* E 56, was grown on a chemically defined medium with glucose and glutamic acid as the sole organic compounds present: neither alone gave rise to the new material. Hydrolysis of culture fluid (8 hr. at 100° with 2 N-hydrochloric acid) did not alter the chromatographic behaviour of the new spot; it was not therefore a peptide. The R_F values were those given for γ-aminobutyric acid by Dent (1948). We considered this sufficient to identify the material as γ-aminobutyric acid since this compound is known to be produced from glutamic acid by some strains of *Bact. coli* (Gale, 1940). Thus it was apparently this decarboxylation which was being recorded on our chromatograms by the disappearance of the glutamic acid and the concomitant appearance of the γ-aminobutyric acid spot. Recently, Woiwod & Proom (1950) read a paper demonstrating chromatographically that *Shigella paradysenterae* produces γ-aminobutyric acid by specific decarboxylation of glutamic acid, and suggested that the corresponding spot in some of their *Bact. coli* filtrates was due to the same cause.

Distribution of γ-aminobutyric acid production. Of the twenty-five strains of

Bact. coli Type I (Wilson, 1935) all produced γ-aminobutyric acid except N.C.T.C. 414. The Eikjman test did not give consistent results with this organism, so that its exact classification was open to some doubt. Results with all the other coliforms were negative save for the anomalous strain E60 which differed from *Bact. coli* in utilizing citrate. All negative results were confirmed by a duplicate experiment in which incubation was continued for 8 days. In our series the production of the γ-aminobutyric acid spot was correlated with a positive Eikjman reaction.

Some experiments on growth in simple chemically defined media were also carried out. Gale (1940), using washed suspensions, found that glutamic decarboxylase was formed only at acid pH. This was usually achieved by adding glucose to the medium, though carbohydrate itself was not necessary for the formation of the enzyme. We found that decarboxylation accompanied growth at pH 5·6 in a heavily buffered medium with glucose and glutamic acid as sole carbon compounds, but not with glutamic acid alone. Growth at pH 7·6 in a heavily buffered medium was not accompanied by decarboxylation save for a very slow reaction in the presence of glucose.

We are much indebted to Prof. T. J. Mackie for his interest in this work, which was performed during the tenure by one of us (H. K. K.) of the Lewis Cameron Teaching Fellowship of Edinburgh University.

REFERENCES

Atkinson, H. F. (1948). Construction of troughs for use in chromatography tanks. *Nature, Lond.*, **162**, 858.

Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). Partition chromatography with paper. *Biochem. J.* **38**, 224.

Dent, C. E. (1948). Paper chromatography of amino-acids. *Biochem. J.* **43**, 169.

Gale, E. F. (1940). Amine formation by *Bact. coli*. *Biochem. J.* **34**, 392.

Linggood, F. V. & Woiwod, A. J. (1948). Application of paper partition chromatography to the production of diphtheria toxin. *Brit. J. exp. Path.* **29**, 283.

Proom, H. & Woiwod, A. J. (1949). The examination, by partition paper chromatography, of the nitrogen metabolism of bacteria. *J. gen. Microbiol.* **3**, 319.

Wilson, G. S. (1935). The bacteriological grading of milk. *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 206.

Woiwod, A. J. (1949). A technique for examining large numbers of bacterial culture filtrates by partition chromatography. *J. gen. Microbiol.* **3**, 312.

Woiwod, A. J. & Proom, H. (1950). *J. gen. Microbiol.* **4**, v.

(*Received* 5 *October* 1949)

A Note on Stalked Forms of Viruses

By C. F. ROBINOW
Department of Bacteriology and Immunology, University of Western Ontario, London, Ontario, Canada

SUMMARY: The filamentous forms of fowl-plague virus, isolated from infected eggs by Elford and Dawson, may be similar in origin to the stalked forms of vaccinia virus discovered in preparations of infected cells by Bland & Robinow, who contended that they were elementary bodies attached to cytoplasmic threads derived from the host cell.

In a study of fowl-plague virus adsorbed on the membrane of laked fowl red cells, Dawson & Elford (1949) found the normal round virus particles associated with hitherto unknown forms described as: '...filaments up to 6 μ. in length and averaging 80 mμ. in width, which in some instances are terminated by a spherical mass 100 mμ. in diameter'. The very clear electron micrograph illustrating Dawson & Elford's discovery, especially the long form extending for about 1·5 μ. beyond the edge of the red cell membrane in that photograph, calls to mind the stalked elementary bodies of vaccinia virus briefly noted by Bland & Robinow (1939) in the following sentence, describing the appearance of rabbit cornea epithelial cells at a late stage of infection with the virus: 'Their surface is often covered with a fringe of short delicate threads of cytoplasm each of which may have a single elementary body forming a little knob at its end (fig. 16*b*).' The structures seen by Bland & Robinow are illustrated in Figs. 1 and 2 of the present note. Both are photographs of epithelial cells in hanging-drop tissue cultures of rabbit cornea infected with vaccinia virus, fixed *in situ* with osmium tetroxide vapour and stained with Giemsa solution. Fig. 1 is here reprinted from our 1939 paper (with acknowledgements to the editors of the *J. Path. Bact.*); Fig. 2 has not been published before. Elementary bodies at the end of short stalks can be seen arising from the free edge of the infected cells. Four of these structures are visible in Fig. 1; two, perhaps three, in Fig. 2. Cells fringed with these stalked forms were frequent in tissue cultures in which the infection had been allowed to run its course.

Dawson & Elford suggested, with due caution, that the long forms of fowl plague may be a phase in the growth of the virus. Their evidence is the similarity of the ordinary and the long forms with regard to their combination with red cells and antibody. Bland & Robinow did not regard the stalked forms of vaccinia virus as a phase in virus multiplication. They interpreted the stalk as an extension of the cytoplasm of the infected cell, with the virus particle forming the knob. Perhaps the stalked forms ultimately break away from the cell surface. Such a course of events, assuming it to be true for fowl-plague virus, would explain the presence of long forms in the allantoic fluid of infected eggs. Studies with the electron microscope of virus-infected tissue cultures would permit the testing of this hypothesis, and would probably be of considerable usefulness in the study of the origin of the long forms of viruses.

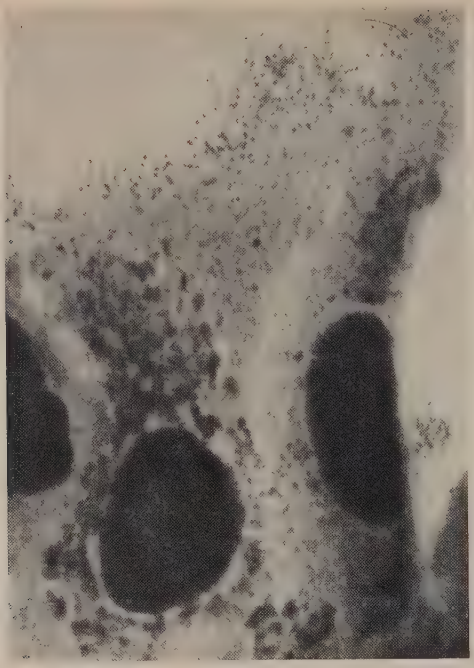
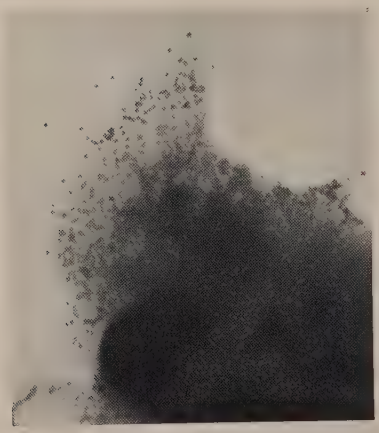


Fig. 1



10 μ

Fig. 2

C. F. Robinow—Stalked forms of viruses. Plate 1

REFERENCES

Bland, J. O. W. & Robinow, C. F. (1939). The inclusion bodies of vaccinia and their relationship to the elementary bodies studied in cultures of the rabbit's cornea. *J. Path. Bact.* 48, 381.

Dawson, I. M. & Elford, W. J. (1949). The investigation of influenza and related viruses in the electron microscope, by a new technique. *J. gen. Microbiol.* 3, 298.

EXPLANATION OF PLATE

Figs. 1, 2. Epithelial cells in tissue cultures of the rabbit's cornea 20 hr. after infection with vaccinia virus. Fixed *in situ* with osmium tetroxide vapour, stained with Giemsa solution. ×1700.

(*Received* 17 *October* 1949)

The Distinction of Licheniformin from Subtilin by Cross-Reactions with Antibiotic-Resistant Strains of *Mycobacterium phlei*

By P. D'ARCY HART and BRENDA MOSS
The National Institute for Medical Research, Mill Hill, London, N.W. 7

SUMMARY: Under standard conditions of subculturing in increasing drug concentrations, licheniformin and subtilin (polypeptide-containing antibiotics from the genus *Bacillus*) induced resistance in *Mycobacterium phlei* more slowly and to a much smaller degree than did streptomycin. Strains resistant to licheniformin and subtilin tended to revert, whereas streptomycin-resistance was stable. Licheniformin resistance was somewhat more difficult to induce and less stable than subtilin resistance.

Cross-resistance tests of bacteriostatic activity between the resistant strains and the three antibiotics distinguished completely licheniformin from both subtilin and streptomycin. Although the difference between licheniformin and subtilin was largely confirmed by a more sensitive bactericidal test, making viable counts after varying periods of exposure of the resistant strains to the antibiotics in nutrient medium, the test, nevertheless, revealed a minor overlap between the two antibiotics, which was either the result of contamination of one by the other or, more probably, the expression of some common chemical property. Licheniformin and subtilin differed in the speed of their lethal action on *M. phlei*, subtilin being much the slower.

When *M. phlei* was exposed to mixtures of streptomycin and licheniformin in certain proportions, the rapid development of high streptomycin resistance was prevented.

Under standard conditions, it is possible to compare the rates of emergence *in vitro* of bacterial strains resistant to different antibacterial substances by repeated subculture of an initially sensitive strain of a suitable bacterium in graded concentrations of these substances. Such resistant cultures can be used for cross-resistance tests, and so serve as a tool for the study of relations between antibacterial substances.

This method was successfully applied to *Streptococcus pyogenes* and *Staphylococcus pyogenes* and various synthetic drugs, including sulphonamides, diamidines and acridines (McIntosh & Selbie, 1943; Wien, Harrison & Freeman, 1948), and to antibiotics like streptomycin and streptothricin, using induced resistant strains of *Staph. pyogenes* (Sullivan, Stahly, Birkeland & Myers, 1946) or *Mycobacterium tuberculosis* (Smith & Waksman, 1947). Schemes for identifying new antibiotics by means of standard resistant strains have been proposed by Eisman, Marsh & Mayer (1946) and by Stansly (1948).

We felt that subtilin (Jansen & Hirschmann, 1944; Lewis, 1947) and licheniformin (Callow, Glover, Hart & Hills, 1947) could be profitably examined by this method, because although distinguished by certain physical and chemical properties, as well as by their range of antibacterial activity, they show some interesting similarities, such as origin from species of the genus *Bacillus* (*B. subtilis* and *B. licheniformis*), basic nature and polypeptide composition, relative thermostability at low pH's, and activity against mycobacteria, including *Mycobacterium tuberculosis*. Streptomycin was also tested to widen the comparison. *M. phlei* was used throughout.

Three main methods were used: (1) measurement of the rate and degree of development of resistant strains to the three antibiotics, and of the stability of these strains; (2) tests of cross-resistance to bacteriostasis; and (3) tests of cross-resistance to killing.

MATERIALS AND METHODS

Antibiotics used. The licheniformin used, containing a mixture of polypeptides of differing activities against mycobacteria, and some inactive impurities (Callow & Work, 1949), was batches 551 and 855, with potencies 5000 and 10,000 units/mg. (Callow *et al.* 1947). Solutions were prepared by dissolving in dil. HCl at pH 2·5 and autoclaving at 10 lb./in.^{2} for 10 min. The subtilin, about 90 % pure (Lewis, 1949), was lot 317, with a potency 2·3 times that of the standard (Fevold, Dimick & Klose, 1948) (in the absence of an agreed unitage, weights are recorded below); solutions were made as for licheniformin and heated for 5 min. in a boiling water bath. The streptomycin was a commercial preparation of the sulphate, potency 900 units/mg. (i.e. *c.* 900 μg. base/mg.); solutions in distilled water were treated as sterile.

The test bacterium. M. phlei was preferred to a virulent *M. tuberculosis* because of its more rapid growth and greater safety. A laboratory strain, P, previously used for routine assays of licheniformin, was chosen. This strain was also sensitive to subtilin and to streptomycin. Both the original strain, to which the designation P is restricted, and all the resistant strains derived from it, were maintained on slopes of Herrold's (1931) egg-yolk agar medium, containing 5 % glycerol. For bacteriostatic tests, suspensions were prepared by grinding the 4–6 days' growth from this medium in Griffith tubes and suspending in distilled water at 0·2 mg. (moist weight)/ml., as judged by opacity comparisons with a standard; such suspensions contained about 25×10^7 viable organisms/ml. The inoculum for 5·5 ml. of the Dubos medium (see below) was 0·2 ml. = 0·04 mg. moist weight, or *c.* 5×10^7 viable organisms. For bactericidal tests, in which periodic viable counts were made, 5·5 ml. of Dubos medium, inoculated as above with 0·04 mg. bacilli, were incubated at 37° for 3 days; the culture was then centrifuged lightly to bring down large clumps, and the upper part of the suspension used. The inoculum was 2·0 ml. for flasks of 55 ml. of the Dubos medium containing the different antibiotics; the resulting mixtures usually contained *c.* 10^6 viable organisms/ml.

Production of resistant strains

In liquid media. The repeated subculture of *M. phlei* P in Hartley's digest broth containing graded concentrations of antibiotic, as was done with subtilin by Salle & Jann (1948), proved unsatisfactory owing to uneven granular growth, particularly near the inhibition end-point concentration. Tween-albumin medium, essentially that of Dubos & Davis (1946), was used thereafter. The basal part of this medium was Difco Casamino acids, 2·5 g.; asparagine, 0·3 g.; anhydrous Na_2HPO_4, 2·5 g.; KH_2PO_4, 1·0 g.; sodium citrate, 1·5 g.; $MgSO_4$, $7H_2O$, 0·6 g.; glycerol, 25 ml.; distilled water to 1 l. It was distributed in 5 ml. quantities in 25 mm. diameter test tubes and autoclaved.

To each tube 0·25 ml. of 5 % albumin (bovine plasma fraction V), filtered through Ford Sterimat SB or through Gradocol membrane 0·56–0·9 μ., and 0·25 ml. of 1 % Tween 80, autoclaved at 15 lb./in.^{2} for 15 min., were added aseptically, making a final volume of 5·5 ml. A standard suspension of strain P was inoculated (in 0·04 mg. quantities) into tubes of this medium containing serial two-fold dilutions of antibiotic, and the lowest concentration giving complete inhibition of growth after 3–6 days' incubation at 37° was taken as end-point of the initial bacteriostatic sensitivity assay. Samples (0·2 ml.) from the tube with the highest concentration of antibiotic permitting definite visible growth were seeded into a new set of tubes, which were incubated, and the process repeated. In later experiments each transfer was made by way of egg-yolk agar and the inocula obtained from this intermediate culture. Thus, when the egg medium culture, grown from a loopful from the appropriate tube, and representing strain P modified by the first exposure to a certain concentration of antibiotic, was 4–6 days old, a standard suspension made from it was again seeded into dilutions of the antibiotic to test sensitivity and to provide the next subculture on egg for the third exposure. The process of transfer and assay was repeated as many times as desired. By this means, the direct transfer of unknown and variable inocula from one series to the next was avoided; the inocula were approximately standard and the sensitivity assays comparable.

On solid medium. The dried surfaces of 9 cm. plates of Hartley's digest broth agar, containing serial dilutions of antibiotic, were seeded uniformly with 0·2–0·5 ml. of a heavy suspension of strain P from egg medium. The plates were sealed, inverted and incubated at 37° for 4–10 days, until good growth was obtained on a control plate. Colonies from the plate containing the highest concentration of antibiotic were subcultured on egg-medium slopes, and the process was repeated.

Bacteriostatic cross-resistance tests

Sets of tubes of Dubos medium (5·5 ml.), containing serial two-fold dilutions of the different antibiotics, received 0·2 ml. of standard suspensions (0·04 mg. bacilli) of the different resistant strains and of strain P, and were incubated at 37°. Inhibitory end-points were read daily for 6 days.

Bactericidal cross-resistance tests

The Dubos basal medium was autoclaved in 50 ml. amounts in 250 ml. conical flasks and 2·5 ml. of sterile albumin and of Tween 80 then added. After introducing the antibiotics the flasks were inoculated with 2·0 ml. of suspensions (p. 245) of the strains under test and incubated at 37°; at intervals during 30 hr. the flasks were shaken and samples taken for viable counts.

For the counts, 9 cm. plates, containing 15 ml. of 5 % horse blood in Hartley's digest broth agar, were used. The dropping-pipette technique of Miles & Misra (1938) was modified in two respects: (1) two separate drops of each dilution were inoculated on each of three plates, instead of one on each of six, the counts being averages of the six drops of a given dilution; (2) the dilutions of the test

fluids containing the bacterial suspensions were made in distilled water instead of in medium or in normal saline; tests showed that over the period (maximum 30 min.) between making the dilutions and inoculating the plates, the lethal effect of water on *M. phlei* (P) was no different from that of saline, both being negligible. After 3–5 days at 37°, usually two of the ten-fold dilutions (10^{-1}–10^{-5}) yielded easily countable colonies and the two counts agreed well. The presence of surface-active Tween 80, and in some cases of subtilin, in the test mixtures made the volume/drop of samples taken for dilution slightly less than that of water; conversion factors were calculated from measurements of these slight differences (see Fildes, 1931), and due allowance made in the readings.

The antibiotic in the fluids sampled did not unduly affect the fluids diluted for the plate-counts. Dubos medium containing 125 units licheniformin/ml. was diluted (10° to 10^{-4}) in water. To 1 ml. of each dilution, and to a drug-free control, was added 0·1 ml. of a 3-day culture of *M. phlei* P in Dubos medium, diluted to give a count of 50–100 organisms/0·02 ml. drop in the control. Immediately each of three blood agar plates was inoculated with a drop of the mixtures. The same procedure was followed with a solution of 200 μg. subtilin/ml. After 3 days' incubation, the average viable counts were:

	Colonies/drop	
Dilution	Licheniformin	Subtilin
10^0	0	0
10^{-1}	56	77
10^{-2}	76	81
10^{-3}	83	61
10^{-4}	73	62
Control	62	75

That is, dilutions of 10^{-1} downwards gave counts approximately equal to those in drug-free controls. The lowest dilution of the test mixtures that could safely be used for plating was therefore clearly 10^{-1}, and this was adopted as a routine.

RESULTS

Production of resistant strains

In Dubos medium, the lowest concentrations of the three different antibiotics completely inhibiting *M. phlei* P were 2 units licheniformin/ml., 3 μg. subtilin/ml. and 0·4 unit streptomycin/ml. Direct transfers were made into new sets of tubes; after twenty-two such transfers in licheniformin-containing medium, in a period of 5 months, the resulting strain was grown on egg medium and its sensitivity compared with that of the unmodified strain P; it was 60 units licheniformin/ml., or 1/30 of the original value for P. Since this ratio was 1/2 after three and 1/16 after eleven of the twenty-two transfers, the rate of decrease was clearly slow. In contrast, the sensitivity to subtilin in Dubos medium decreased to 1/30, and to streptomyein to 1/5000, of the initial values for strain P after three transfers.

To improve comparability, the use of intermediate egg-yolk agar cultures as a stage in the transfers, with standardization of suspensions prepared therefrom

for inoculation (p. 245), was adopted in a concurrent comparison of the rates of production of resistant strains in Dubos medium containing increasing antibiotic concentrations, starting again from strain P. Fig. 1 records the sensitivities of each of the strains after five exposures to the three antibiotics,

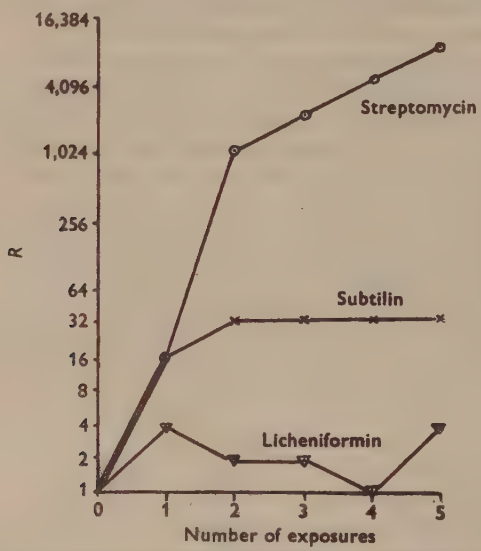


Fig. 1. Increase in resistance of *M. phlei* to licheniformin, subtilin and streptomycin, after repeated subculture in graded concentrations of antibiotic. R = ratio of the minimum inhibiting concentrations of the antibiotics to the initial minimum inhibiting concentrations, which were licheniformin, 2 units/ml.; subtilin, 3 μg./ml.; and streptomycin, 0·2 unit/ml.

expressed as inverse ratios to the corresponding initial sensitivities of the unmodified P. It is clear that streptomycin-resistance increases rapidly to its very high value of more than 10,000-fold, whereas licheniformin and subtilin resistance reach limits at levels not much above the initial values.

In case the feeble licheniformin resistance was due to the medium, attempts were made to induce resistance on agar plates. In the first set of plates there was no growth on agar containing 200 units licheniformin/ml., but 100 colonies on 100 units/ml. After eight repetitions a small amount of growth was visible on the plate containing 5000 units/ml. In a similar experiment with streptomycin, one colony grew on the plate containing 1000 units/ml., even in the first set.

It is more difficult to maintain on nutrient agar a bacterial population resistant to licheniformin than a population resistant to streptomycin. Licheniformin, subtilin and streptomycin were incorporated in 9 cm. broth agar plates at concentrations designed to cover a similar range. One-half of the surface of each plate was uniformly seeded with a light suspension of *M. phlei* (0·04 mg. moist weight) resistant to the given antibiotic, and the other half with a heavy suspension (0·4 mg.), and the plates were sealed and incubated. The suspensions

were from egg-medium cultures of strains insensitive to 5000 units licheniformin/ml., 500 units streptomycin/ml. and 1000 μg. subtilin/ml., respectively. Table 1 shows the growth after 7 days. It needed a heavy inoculum of bacteria previously resistant to 5000 units licheniformin/ml. to establish growth at the same concentration, and even at 100 units/ml. growth was not profuse; light inocula failed to grow at the higher concentration and grew only slightly at the lower. With streptomycin-resistant bacteria, on the other hand, even a light inoculum grew as profusely at 1000 units/ml. as it did on the control plate.

Table 1. *Growth from suspensions of* M. phlei *previously resistant to licheniformin, subtilin and streptomycin, seeded on nutrient agar plates containing graded concentrations of the same respective antibiotics*

Bacterial suspension	Antibiotic to which *M. phlei* had been resistant, and against which it was re-tested: Licheniformin (units/ml.) 5000	100	10	0	Subtilin (μg./ml.) 1000	10	1	0	Streptomycin (units/ml.) 1000	10	1	0
Light	0	±	+++	+++	±	+++	+++	+++	+++	+++	+++	+++
Heavy	Tr.	++	+++	+++	+	+++	+++	+++	+++	+++	+++	+++

Tr., ±, +, ++, +++ indicate degrees of growth.

The behaviour of subtilin-resistant organisms was intermediate. In the case of licheniformin, therefore, and to a lesser extent of subtilin, highly resistant strains were difficult to maintain and had partially reverted to sensitive, in sharp contrast to the stability of the streptomycin-resistant strain.

In another comparison of stability, resistant colonies from broth agar containing high concentrations of the three antibiotics were subcultured on egg medium, and standard suspensions re-tested against the same antibiotics in the Dubos medium. The licheniformin-resistant organisms now resisted only 8 units licheniformin/ml. instead of 5000 units/ml. previously in agar; the subtilin-resistant strain 64 μg. subtilin/ml. instead of 1000 μg./ml.; but the streptomycin-resistant organisms were still resistant to 1000 units streptomycin/ml. The resistances in Dubos medium were 8, 40 and 10,000 times those of the unmodified strain P tested at the same time. The contrast between resistance to licheniformin and to streptomycin, with subtilin resistance intermediate, is again evident.

Cross-resistance tests

Bacteriostatic test. The sensitivity of licheniformin-resistant, subtilin-resistant and streptomycin-resistant *M. phlei* (being the fifth transfers in the experiment illustrated in Fig. 1), as well as that of strain P from which they were derived, was measured simultaneously against each of the three antibiotics in Dubos medium. Table 2 gives the 3-day readings in one experiment. The resistance of a modified strain to an antibiotic is indicated by the ratio of the bacteriostatic titre of the antibiotic against this strain to its activity against the original, unmodified strain; a difference less than four-fold indicates

Table 2. *Minimal inhibiting concentrations* of three antibiotics for induced resistant strains of* M. phlei

Antibiotic	Modified strains: Streptomycin-resistant	Modified strains: Licheniformin-resistant	Modified strains: Subtilin-resistant	Original unmodified strain P
Streptomycin	>1000	0·4	0·4	0·2
Licheniformin	2	8	4	2
Subtilin	nt	6	100	12·5

* In units/ml. of Dubos liquid medium for streptomycin and licheniformin, and in μg./ml. for subtilin. nt = no test.

susceptibility and a four-fold or greater difference, resistance. The contrasts that then emerge are: (1) the highly streptomycin-resistant strain was susceptible to licheniformin, and the slightly licheniformin-resistant strain was susceptible to streptomycin; (2) the moderately subtilin-resistant strain was susceptible to licheniformin, and the slightly licheniformin-resistant strain was susceptible to subtilin. There is no evidence of cross-resistance or of overlapping activity, and on this basis licheniformin is distinguished both from streptomycin and from subtilin. The first of the two contrasts would be sharper if licheniformin resistance were of a higher order, but even so it is probably significant. On the other hand, the distinction between licheniformin and subtilin is less marked owing to the relatively low resistances to both these antibiotics demonstrable by the method.

Bactericidal tests. The drawbacks of cross-resistance tests where bacteriostatic activity is measured, led us to use bactericidal tests in the same medium. Since streptomycin is bactericidal in nutrient media (Garrod, 1948), it was anticipated that subtilin and licheniformin might also have this property. If the resistance induced to lethal action of the antibiotics were greater than that induced to bacteriostatic action, the cross-resistance tests would give sharper contrasts. Preliminary tests showed the useful bactericidal concentrations to be 12·5 and 125 units licheniformin/ml., 20 and 200 μg. subtilin/ml., and 12·5 units streptomycin/ml. Incidentally, on a weight basis, subtilin killed strain P much more slowly than did licheniformin; moreover, the ratio of the concentrations of streptomycin and licheniformin of equal bactericidal potency was the same as the ratio of concentrations of equal bacteriostatic potency, whereas with subtilin and licheniformin the bactericidal ratio was considerably greater than the bacteriostatic ratio. These observations suggest that the action of subtilin and licheniformin differ.

Licheniformin-resistant (LR) and subtilin-resistant (SR) *M. phlei*, as well as the strain P from which they were derived, were exposed to licheniformin and to subtilin, with suitable controls. The resistant strains had grown in the presence of 5000 units licheniformin/ml., and 1000 μg. subtilin/ml., in broth agar. The concentrations of licheniformin and subtilin in the flasks were 12·5 units/ml. and 20 μg./ml., respectively, of Dubos medium. In Fig. 2 A–F, $\log_{10}$ counts are plotted against time. Points based on mean counts of less than one colony per drop are bracketed, and points based on zero mean counts

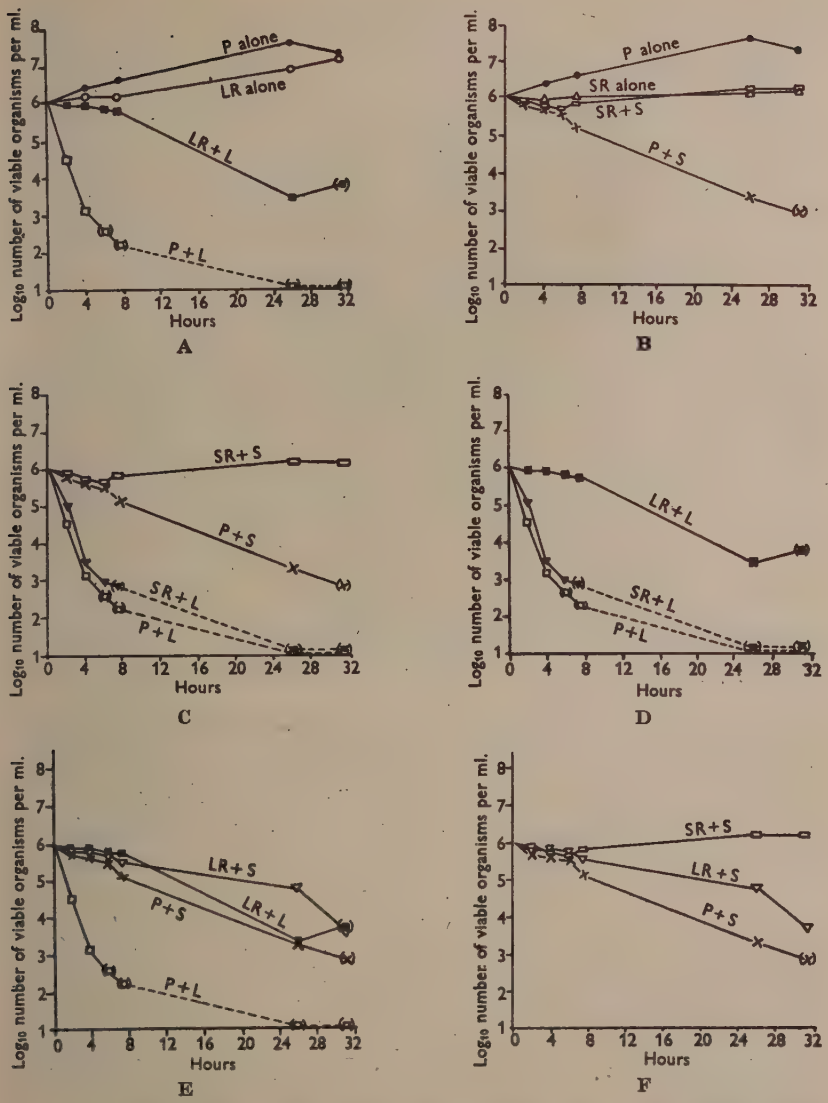


Fig. 2A–F. Viable counts in bactericidal tests of licheniformin and subtilin on induced resistant strains of *M. phlei* in Dubos liquid medium. L = licheniformin; S = subtilin; P = unmodified strain; LR = licheniformin-resistant strain; SR = subtilin-resistant strain. All curves are adjusted to a 0 hr. reading of 6·0. The initial log counts were in fact: P, 5·7; LR, 6·8; SR, 6·1; P+L, 5·7; LR+L, 6·8; SR+L, 6·0; P+S, 5·8; LR+S, 6·8; and SR+S, 6·0. Brackets indicate readings, and interrupted lines, portions of curves, of doubtful reliability (p. 252).

per drop of the lowest dilution used (i.e. no colonies from any of the six drops plated from a 10^{-1} dilution) are placed arbitrarily on the base-line and connected by interrupted lines, to indicate doubtful reliability. The curves have been adjusted, for convenience, to a common starting-point of 6·0 (i.e. initial count of 10^6 viable organisms/ml.); this was considered justifiable because in a number of tests on each type of mixture, with initial log counts from 4 to 7 as the sole variable, the shape of the curves was found to be similar, and because most initial log counts fell between 5·5 and 6·5. The trends of the curves, rather than a comparison of the individual points on them, are the relevant consideration. Resistance of a modified strain to an antibiotic is indicated by the divergence of the curve for the behaviour of this test-mixture and that for strain P exposed to the same antibiotic.

The rapid lethal action of licheniformin on strain P is evident (Fig. 2A); in the strength used, it also killed the LR strain, though the rate was slow in comparison, especially in the first hours. The curves for P and LR alone were similar. Subtilin killed P slowly (Fig. 2B), but since the SR strain was hardly affected by this concentration (the curve, after an initial slight fall, rises slowly, rather like the curve for SR alone), a difference in bactericidal effect was manifest. In other experiments the course of the curves for P and SR alone was similar. From these observations it is clear that bactericidal tests revealed resistance to the inducing antibiotic in the LR and SR strains used. Larger antibiotic concentrations in other experiments gave no advantage, but, on the contrary, increased the downward gradient of curve LR + L more than that of P + L and so brought the two curves nearer together; the effect on curves SR + S and P + S was similar.

The following contrasts appear from the results of the cross-resistance tests: (1) The SR strain, which was resistant to the lethal effect of subtilin, was almost, if not fully, susceptible to licheniformin, the contrast being shown by the proximity of curves SR + L and P + L and the divergence of curves SR + S and P + S (Fig. 2C); this susceptibility also contrasts with the resistance of the LR strain to licheniformin (Fig. 2D). (2) The LR strain, which was resistant to the lethal effect of licheniformin, was also killed rather more slowly than strain P by subtilin, but comparison of the divergence of curves LR + S and P + S with that of LR + L and P + L shows the cross-resistance of LR to subtilin to be less than its resistance to the inducing antibiotic (Fig. 2E); it is also less than the resistance of the SR strain to its inducing antibiotic (Fig. 2F).

Whether comparison be made of the bactericidal effects of the two antibiotics on the same resistant strain, or of the same antibiotic on the two resistant strains, licheniformin and subtilin can, therefore, be differentiated. There is some evidence of cross-resistance and overlapping activity, but of a minor degree. Other experiments, using the same or ten times the concentrations of antibiotics, supported these conclusions.

Comparison between streptomycin and licheniformin was limited to an experiment in which a streptomycin-resistant strain (SmR) and the unmodified strain P were exposed to each of these antibiotics. The resistant strain had grown in the presence of 250 units streptomycin/ml. in Dubos medium. The

concentrations of streptomycin and licheniformin in the flasks were 125 units/ml. and 12·5 units/ml., respectively. As might be expected, there was a marked divergence between the course of the SmR + Sm curve, which, like the curve for SmR alone, rose gradually, and the course of the P + Sm curve, which fell rapidly to give zero counts in 6 hr.; streptomycin resistance of the SmR strain was thus clearly demonstrable. Against this resistance the susceptibility of the SmR strain to licheniformin, shown by the close proximity of curves SmR + L and P + L, stood in strong contrast, thus clearly distinguishing the two antibiotics. The distinction is not complete since no test of the effect of streptomycin on LR bacteria was made.

Production of resistant strains to antibiotic mixtures

The slight cross-resistance between the licheniformin-resistant and subtilin-resistant strains might be accounted for by contamination of one antibiotic with the other. To examine this possibility, resistant strains were induced by mixtures of antibiotics. For technical convenience streptomycin was selected

Table 3. *Tests of cross-resistance of strains made resistant by exposures to streptomycin-licheniformin mixtures*

		Antibiotic used for test (ratio min. inhib. conc. for resistant strains to value for unmodified strain P)			
Antibiotic used for inducing resistance	No. of exposures	Streptomycin + licheniformin (1:1*)	Streptomycin + licheniformin (9:1)	Licheniformin	Streptomycin
Streptomycin + licheniformin (1:1)	7	4	—†	4	8
Streptomycin + licheniformin (9:1)	4	—	64	4	>500
Licheniformin	5	—	—	4	2
Streptomycin	2	—	—	1	>500

* Proportions are by weight.
† Not done.

because it induces resistance easily, and licheniformin because it does so with difficulty. The procedure was one used previously for the single antibiotics: exposure of strain P to graded concentrations in Dubos medium, with intermediate transfers on egg-yolk agar. Mixtures of streptomycin and licheniformin were prepared, in proportions of 1:1 and 9:1 by weight (i.e. 1:10 and 9:10 in units), from two individual solutions at 1 mg./ml., and were serially diluted as usual. Table 3 records the sensitivities of the resulting strains to the different mixtures, and to their separate components. The strain induced by the 1:1 mixture was slightly resistant both to this mixture and to licheniformin (the inhibitory concentration being in each case four times that for strain P), and the resistance was similar to that of the LR strain control to licheniformin. Although this strain had a slight resistance to streptomycin (the inhibitory concentration being eight times that for strain P), it was much less than that

of the SmR strain control (a ratio of >500). The strain induced by the 9:1 mixture also was slightly resistant to licheniformin, though this was considerably less than its resistance to the mixture itself; on the other hand, this strain, like the SmR strain, was highly resistant to streptomycin. Having regard to the susceptibility of the LR strain to streptomycin and of the SmR strain to licheniformin, cross-resistance is thus evident between the strain induced by each antibiotic mixture and that induced by each of the two components singly; in the case of the 1:1 mixture the overlap is mainly with licheniformin, and in that of the 9:1 mixture more with streptomycin, though in the latter case even the small amount of licheniformin exerted a detectable effect. Certain combinations of antibiotics, therefore, can, in cross-resistance tests, display the characteristics of one component predominantly and of the other to a minor degree. We recognize that this experiment suffers from being confined to bacteriostatic measurements; no bactericidal tests were made.

DISCUSSION

The ability of antibiotics to induce resistant strains in bacteria varies greatly. Streptomycin-resistant strains can be produced very easily. It seems more difficult, in general, to produce bacterial cultures resistant to the polypeptide substances derived from the genus *Bacillus*, at least *in vitro*, though variation is evident also within this group. Moderate resistance is induced fairly rapidly by subtilin (Salle & Jann, 1948; and the present work). The same is true for aerosporin and polymyxin with some susceptible bacteria, but with others the increase in resistance is only slight, even after many exposures (Brownlee & Bushby, 1948; Jawetz & Coleman, 1949). Our experiments have shown that, in *M. phlei*, licheniformin resistance is somewhat more difficult to induce than subtilin resistance under comparable conditions, both contrasting strongly with streptomycin resistance. Similarly, after subculture of strains isolated from high concentrations of these three antibiotics, streptomycin resistance is stable, while licheniformin-resistant strains partially revert; subtilin resistance is again intermediate. These differences in rate and degree of emergence of resistant strains to licheniformin, subtilin and streptomycin, and in their stability, suggest that these antibiotics are inherently distinct.

Cross-resistance tests in which bactericidal activity is measured by viable counts have proved, in our hands, more precise in the comparison of licheniformin and subtilin than have bacteriostatic tests; and it may be that this technique would be generally useful where the antibiotics whose relationships it is desired to study induce relatively slight degrees of resistance to themselves as judged by reduced bacteriostatic activity. Against the background of more clearly distinguishable resistance to *killing*, by the inducing antibiotic, bactericidal cross-resistance tests revealed a minor degree of overlap between licheniformin and subtilin, but still indicate substantial differentiation. The distinction is further supported by the much slower rate of killing of the unmodified *M. phlei* by subtilin than by licheniformin. It agrees with salient physical and chemical evidence (Callow *et al.* 1947). It is more difficult to

account for the slight cross-resistance. Contamination of one antibiotic by another could produce such an effect, as has been shown here using streptomycin-licheniformin mixtures as a convenient model; but current chemical work on licheniformin offers no support for the hypothesis that this antibiotic contains even small quantities of subtilin (Callow & Work, 1949). The slight overlap found between licheniformin and subtilin is, therefore, more likely to be a reflexion of a chemical property, e.g. certain amino-acids, in common.

The results of inducing resistance in *M. phlei* to streptomycin-licheniformin mixtures have an incidental additional interest. When the proportion is 9:1 the induced strain has as high a resistance to streptomycin as when the latter is used singly, whereas the strain induced to the 1:1 mixture is only slightly streptomycin resistant. Graessle & Pietrowski (1949) have shown that *M. tuberculosis* exposed similarly to streptomycin together with *p*-aminosalicylic acid, in a proportion of about 2:1, results in delay of the development *in vitro* of streptomycin resistance—a finding with possible clinical application.

We are indebted to Dr R. K. Callow for the licheniformin and to Dr H. Lineweaver, Western Regional Research Laboratory, California, for the subtilin.

REFERENCES

Brownlee, G. & Bushby, S. R. M. (1948). Chemotherapy and pharmacology of aerosporin. *Lancet*, i, 127.

Callow, R. K., Glover, R. E., Hart, P. D'Arcy & Hills, G. M. (1947). Licheniformin, an antibiotic substance from *Bacillus licheniformis*, active against *Mycobacterium tuberculosis*. *Brit. J. exp. Path.* **28**, 418.

Callow, R. K. & Work, T. S. (1949). Personal communication.

Dubos, R. J. & Davis, B. D. (1946). Factors affecting the growth of tubercle bacilli in liquid media. *J. exp. Med.* **83**, 409.

Eisman, P. C., Marsh, W. S. & Mayer, R. L. (1946). Differentiation of antibiotics by resistant strains. *Science*, **103**, 673.

Fevold, H. L., Dimick, K. P. & Klose, A. A. (1948). Isolation of subtilin from submerged cultures. *Arch. Biochem.* **18**, 27.

Fildes, P. (1931). Medical Research Council's *System of Bacteriology in Relation to Medicine*, **9**, 174. London: H.M. Stationery Office.

Garrod, L. P. (1948). The bactericidal action of streptomycin. *Brit. med. J.* i, 382.

Graessle, O. E. & Pietrowski, J. J. (1949). The *in vitro* effect of *para*-aminosalicylic acid (PAS) in preventing acquired resistance to streptomycin by *Mycobacterium tuberculosis*. *J. Bact.* **57**, 459.

Herrold, R. D. (1931). Egg yolk agar medium for the growth of tubercle bacilli. *J. infect. Dis.* **48**, 236.

Jansen, E. F. & Hirschmann, D. J. (1944). Subtilin—an antibacterial product of *Bacillus subtilis*: culturing conditions and properties. *Arch. Biochem.* **4**, 297.

Jawetz, E. & Coleman, V. R. (1949). Laboratory and clinical observations on aerosporin (Polymyxin B). *J. Lab. clin. Med.* **34**, 751.

Lewis, J. C. (1947). Chemistry of subtilin. *Amer. Ass. for Advancement of Science, Symposium on Antibiotics*. Chicago.

Lewis, J. C. (1949). Personal communication.

McIntosh, J. & Selbie, F. R. (1943). The production of drug-resistant cultures of bacteria *in vitro* and a study of their inter-relationships. *Brit. J. exp. Path.* **24**, 246.

Miles, A. A. & Misra, S. S. (1938). The estimation of the bactericidal power of the blood. *J. Hyg., Camb.*, **38**, 732.

Salle, A. J. & Jann, G. J. (1948). Studies on subtilin-fastness *in vitro*. *J. Bact.* **55**, 463.

Smith, D. G. & Waksman, S. A. (1947). Tuberculostatic and tuberculocidal properties of streptomycin. *J. Bact.* **54**, 253.

Stansly, P. G. (1948). Identification of antibiotics by means of resistant strains of bacteria. *J. Bact.* **55**, 721.

Sullivan, M., Stahly, G. L., Birkeland, J. M. & Myers, W. G. (1946). Reciprocal sensitivities of *Staphylococcus aureus* to streptomycin, streptothricin and penicillin. *Science*, **104**, 397.

Wien, R., Harrison, J. & Freeman, W. A. (1948). Diamidines as antibacterial compounds. *Brit. J. Pharmacol. & Chemotherap.* **3**, 211.

(*Received* 20 *October* 1949)

The Morphology and Motility of *Proteus vulgaris* and Other Organisms Cultured in the Presence of Penicillin

By A. FLEMING, AMALIA VOUREKA, I. R. H. KRAMER and W. H. HUGHES

The Wright-Fleming Institute of Microbiology, London, W. 2

SUMMARY: Microbes were grown on microscope slides so that the growth could readily be observed by phase-contrast microscopy.

Proteus vulgaris, grown on agar containing penicillin, undergoes extraordinary morphological changes which vary with the temperature of incubation, the concentration of the penicillin, the concentration of the agar and the presence of small amounts of fluid between the agar and the cover-slip. The bacilli may divide normally once or twice into elements that grow without dividing and which may develop into fantastically shaped thread or swollen forms. In high concentrations of penicillin the fantastic shapes are obtained by enlargement without division. At first the nuclei divide as in normal organisms. The thread forms have condensed nuclei arranged in alternating pattern along the side of the cells. In the swellings there may be either nuclear material filling the cells, a condensed central mass or a reticulum. When vacuoles are present these displace the nuclear material.

When the misshapen organisms are transferred to a medium free from penicillin and containing penicillinase they divide, forming normal bacilli. Many of the swollen elements burst and disappear.

The motility of the greatly enlarged organisms is sluggish, and flagellar movement can clearly be observed by phase contrast. The movement of the flagella of the organisms responds readily to radiant heat, and a careful study of these movements makes it impossible to accept Pijper's contention that bacterial motility is due entirely to undulatory movements of the body and that the flagella are merely mucoid strands cast off as the result of motility.

The flagella were demonstrated in the large forms by fixing the culture through the agar for several days, detaching the agar and staining the cover-slip, which carries the fixed colony, with a saturated watery solution of night blue. The nuclei were shown by treating films with hot nitric acid, washing and staining first with cresyl blue then Leishman's stain.

It was pointed out by Gardner (1940) that bacteria grown in non-lethal concentrations of penicillin exhibit a grossly modified morphology resulting apparently from a failure of cell division. Among the cocci swollen and bloated forms appear and the bacilli form long threads. Gardner's observations have been confirmed by many observers. Similar changes, but perhaps not so extensive, have been noted after exposure of bacteria to other chemicals (Ainley Walker & Murray, 1904; Spray & Lodge, 1943; Webb, 1948; for a review of the literature see Klieneberger-Nobel, 1949).

None of the workers, however, has illustrated clearly the completeness of the morphological changes which can be induced by penicillin on relatively insensitive organisms like *Proteus vulgaris*.

P. vulgaris is an easy organism to work with; it grows rapidly at room

temperature and with penicillin grows in the most fantastic forms. Moreover, it is a highly motile organism possessing many flagella and serves well for a study of the functions of these flagella.

MATERIALS AND METHODS

Microscopy. Phase-contrast microscopy has enabled us to observe clearly the growth of *P. vulgaris* on agar containing penicillin in various concentrations. Much more dramatic results were obtained with cultures on agar than with cultures in a fluid medium, and there was a great difference between cultures grown at 37° and at room temperature (18–20°). At the lower temperature the changes took place more slowly: there was more bacterial growth and in many ways the morphological changes were clearer. The cultures at room temperature were much more valuable for studying motility and flagellar action.

For the most part cultures were grown on microscope slides and were examined by phase contrast. Under a $\frac{1}{6}$th dry objective the culture could be examined repeatedly or continuously without even the slight disturbances which might result if an oil immersion lens was used. For the observation of flagella, however, a $\frac{1}{12}$ in. oil immersion was necessary.

Slide-culture technique. Slide cultures for continuous examination were made in two ways:

(1) Penicillin agar was spread in a thin layer on a sterilized microscope slide. The slide was heated and with a pipette the melted agar distributed as evenly as possible over the central portion of the slide. When the agar had set it was trimmed to a square with the edge of another slide so that it was a little smaller than the cover-slip to be used. A small loopful of a dilution of a young *P. vulgaris* culture was placed on the agar, and a sterile cover-slip applied. Small irregularities on the surface could, if desired, be made before the agar was inoculated, so that it was possible to observe the bacilli: (*a*) with the cover-slip in direct contact with the agar; (*b*) with a very thin layer of fluid between the cover-slip and the agar in which the bacilli had some free movement; and (*c*) a deeper pool of fluid in which the movements were quite unhindered.

On removing the cover-slip from such a culture there was an enormous disturbance of the bacteria so that it was unsuitable for the preparation of permanent stained specimens. When permanent specimens of the undisturbed culture were required, or when we wished the whole of the agar to be in contact with the cover-slip, the preparations were made as follows:

(2) The culture is spread on a sterile cover-slip and allowed to dry. Then penicillin agar (1–1·5 % agar) at a temperature under 50° is dropped on the cover-slip and as soon as it has set the cover-slip is inverted on a slide. When the cover-slip is picked up with a pair of forceps the whole of the agar adheres to it and there is almost no disturbance of the culture. The cover-slip with the agar can be fixed in formalin and when fixation is complete the agar can be gently removed leaving on the cover-slip an almost undisturbed pattern of the

bacilli in the culture which can be stained to give a beautiful permanent record of the culture.

In addition to simple staining the bacilli were treated to show their nuclei and their flagella.

Flagella staining. Slide cultures made by method (2) were used. They were observed by phase contrast and if flagella were seen the cover-slip with the agar attached was picked off and placed in formalin in a Petri dish. After 2 days' or more fixation the agar was gently removed from the cover-slip. This was washed to remove all traces of the culture medium and was then stained. Kirkpatrick's staining method gave fairly good results and when this was followed by staining with carbol fuchsin for 5 min. the flagella were much more prominent.

Later we found that simple watery solutions of certain dyes stained the flagella well after prolonged formalin fixation and the best we found to be a saturated watery solution of night blue applied for 5 min. at room temperature.

Nuclear staining. The demonstration of nuclear material was long impeded by the staining affinity of the cytoplasm for basic dyes after fixation. Various methods have overcome this disadvantage (see Robinow, 1945).

Another acid hydrolysis staining technique was devised by one of us (I. R. H. K.) and used to study the distribution of nuclear material in our cultures.

Film or impression preparations are air-dried. A Petri dish is filled with a 7 % v/v dilution of concentrated nitric acid in distilled water. This is heated to boiling point and allowed to cool. When cold, the slide is placed in the solution, which is again heated until 'bumping' starts. The slide is at once removed from the acid and washed for 15–30 sec. in running tap water. Excess water is shaken off and the slide flooded with 1 % brilliant cresyl blue in 0·9 % saline. After 15–30 sec. this solution is washed off with tap water, the slide rinsed with distilled water, and flooded with Leishman's stain which is then diluted with an equal volume of distilled water. Staining is usually complete in 30 sec. or less, when the slide is washed with distilled water and blotted dry or mounted wet. The nuclear material is stained blue-black and the rest of the cell pink. The intensity of the counter-staining is increased by prolonging the washing in distilled water before the Leishman solution is applied. It has been found that with some batches of brilliant cresyl blue, more selective staining is obtained if the solution has been kept for a few months.

RESULTS

The effect of penicillin on growth and morphology

High concentrations of penicillin in agar. The strain of *P. vulgaris* used in these experiments was, after 24 hr., completely inhibited by 12·5 units (u.) of penicillin/ml.

Experiments were made with concentrations in the agar of from 1 to 3000 u./ml. The results varied greatly with the concentration but even with 3000 u./ml. there was evidently some growth, although there was no cell

division. Some organisms enlarged to 50 or 100 times their normal size, became rounded and swollen and resembled some strange protozoon rather than a bacterium.

The most common forms obtained with different concentrations of penicillin are illustrated in Fig. 1.

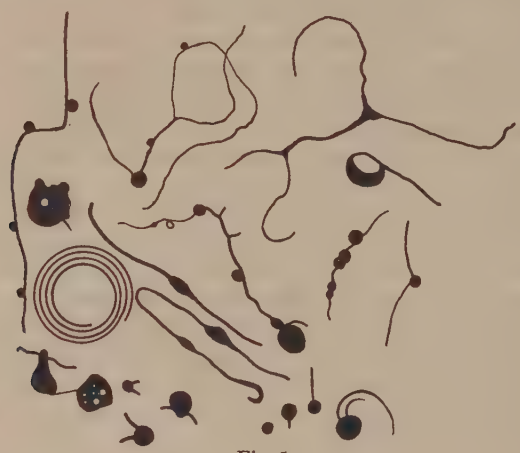


Fig. 1

Low concentrations of penicillin in agar. According to the physical conditions (strength of agar, presence of fluid, etc.) there were great variations in the morphology of the organisms. Pl. 1, figs. 1–6, which are photographs of the same bacteria during a period of 19 hr. at room temperature, gives a fairly typical picture of the development of a bacillus where the agar and the cover-slip were in apposition. The organism, after a period of 1–1½ hr., divides and the daughter cells appear normal. These may again divide producing normal or somewhat larger cells. After one or two normal divisions, however, the cells elongate without division and this elongation may be very great. Very frequently these long forms coil themselves into watch-spring-like spirals and rotate slowly or rapidly without changing their position in the microscopic field (Pl. 1, figs. 4, 5; Pl. 3, fig. 28).

Meanwhile, bulbous enlargements develop in many of the rods (Pl. 1, fig. 5). There may be one or many along the length of the filament, lying centrally in the axis of the filament or forming excrescences on one side or other. Often when a spiral form develops a large bulb, it rotates with this bulb as its axis as though the bulb had made a depression in the agar so that the organism was anchored except for a rotary movement with the bulb at the centre. These bulbs remain small, or enlarge until they are 20 or more times the diameter of the filament, which itself is 2 or 3 times the diameter of the normal bacillus.

There is a great variation in the individual organisms. Some grow into filaments with or without bulbous enlargements; some develop bulbs without much elongation and some are perfect spheres which may be actively motile.

Where there is free fluid many of the organisms do not appear to change. It is common to see practically all the organisms misshapen where the agar is in contact with the cover-glass, but round the edges where there is free fluid there are myriads of actively motile and apparently normal bacilli mixed with a few abnormal forms.

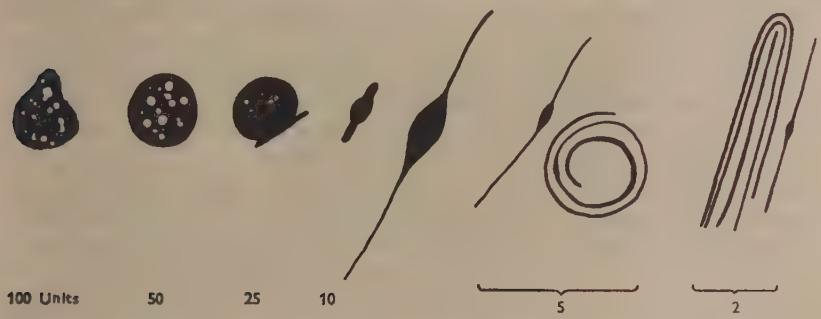


Fig. 2

With higher concentrations of penicillin, up to 25 u./ml., there is perhaps one normal division and then cells elongate into filaments with or without bulbs or develop bulbs with little or no filamentous expansion. When there is a very thin layer of fluid under the cover-slip it is not uncommon to find extraordinary branched forms (Pl. 3, fig. 26). These are sluggishly motile and often only one branch may be moving at one time.

With still higher concentrations of penicillin there is no division and many of the organisms remain unchanged; but many, on the other hand, develop into large bulbs perhaps with one or two short projections representing the end or ends of bacilli (Pl. 3, fig. 27). Fig. 2 illustrates some of the bizarre forms which may be seen in 24 hr. slide cultures at room temperature.

Imitation of bathing pool or sun-bathing beach. Apart from the activity of the individual organisms certain activities on the part of the group may be noticed. When a low concentration of penicillin (say 5 u./ml.) is used in the agar and there is a slight irregularity of the surface so that a shallow pool with a shelving edge is formed under the cover-glass, a very good imitation is seen of a sun-bathing beach. The shelving edge is covered with radially arranged long bacilli, just like people stretched out on the sands. Every now and then one swims off into the pool and its place is taken by another. This is best seen before the organisms have developed bulbs, otherwise the bulbous ones seem to become anchored and cannot leave the beach. Pl. 2, fig. 18, illustrates such a pool. Here many of the organisms actually swimming in the fluid are bulbous. They are too large to get back to shore.

Penicillin-induced change in nuclear material

There is a regular sequence of development of the abnormal forms, and though it does not take place at the same rate in all the bacilli the order of stages appears to be constant. The general morphology of the types has been

described; the flagella are dealt with below; the nuclei at the various stages are as follows.

Stage 1. *The small cells* have nuclei in the positions described by Robinow (1945) for normal cells; but these lose their dumb-bell form and become rounded (Pl. 2, figs. 7–9).

Stage 2. *Long filaments* have nuclei similar in appearance to those of normal cells but repeated along the threads. All stages of division may be seen in one thread, and dumb-bell forms are more easily distinguished. In some threads, probably those more affected or exposed for longer periods, nuclear division ceases, the nuclei condense into dots which are often arranged along the thread on alternate sides, in contact with the cell membrane.

Stage 3. *Threads with swellings*. The nuclei of the threads are similar to those of organisms in stage 2. In the swellings several different nuclear appearances are found. The nuclear material may fill the space (Pl. 2, fig. 13), be collected into a central mass (fig. 14) or form a coarse reticulum (fig. 18).

Stage 4. *Spheres*. These have the same type of nuclei as stage 3 or swellings (Pl. 2, fig. 16). When vacuoles are present these displace nuclear material (Pl. 2, fig. 17).

Recovery from exposure to penicillin

Pl. 3, figs. 22–25, give the sequence of events when organisms from the culture on penicillin agar in the series (Pl. 1, figs. 1–6) were transferred to agar containing penicillinase. The cover-slip was lifted from the block of penicillin-agar and with the adherent organism transferred to a block of penicillinase-agar, resealed and the second series of photographs made.

Pl. 3, fig. 22, shows organisms of various shapes. After transferring to the penicillinase-agar the fusiform organism develops back into normal organism, separation of the ends taking place first. Next the bulbs divide across to give two club-shaped cells which in subsequent divisions approximate more and more to the normal bacillary forms.

The division of the filament and the multiplication of the young organisms derived therefrom results in a collection of normal bacilli round the large swellings, which burst and degenerate. But for the continuous observation the appearance might have suggested that the small forms were liberated from the bursting of a cyst-like formation. Nevertheless, organisms derived from spheres may occur.

Motility and flagella in penicillin-induced forms

Traditionally flagella are regarded as the organs of motility in bacteria, but recently Pijper (1946) has maintained that motility is due to 'gyratory undulating' movements of the bacillary body and the flagella are 'slime layer' strands thrown off because of the active motion. When one observes the short active bodies of *Salmonella typhi* or *Proteus vulgaris* the movement is too rapid for any conclusion about the nature of motility to be drawn. The case is quite different, however, with the large filamentous and bloated forms of *P. vulgaris* grown in slide culture on penicillin agar. The activity of the organisms depended to some extent on the density of the agar and whether there was fluid between

the cover-slip and the agar. When the agar was very soft or when there was the thinnest layer of fluid under the cover-slip, then at some stage, all or most of the organisms were motile at room temperature. When the agar was stiff and there was no fluid a large proportion of them were non-motile, especially when grown at 37°.

In all cases the motility of these enlarged organisms was sluggish compared with normal *P. vulgaris* in a fluid medium, and the flagella could be observed directly by phase-contrast microscopy. Individual flagella were probably not visible but in these gross forms, when the filaments were close together, the flagella formed themselves into wavy rope-like strands which could easily be seen (Pl. 3, fig. 28).

When there was a considerable space between the filaments a wavy motion could be seen in the fluid indicating flagellar activity, although the actual flagella could not be seen.

The effect of radiant heat on motility. The motility of these organisms is very sensitive to heat. Heat filters which hardly affect the intensity of the light from the microscope lamp, stop motility when placed across the beam of light. This effect is not evident in the very active younger cultures nor is it seen in all the motile bacteria of a culture. It is most marked in cultures 24 hr. or more old, grown at room temperature, in which the motility is more sluggish.

The fact that these organisms exhibited active movements in the slide cultures and yet remained in the same microscope field made it easy to follow the effect of heat on their movements. Without a heat filter they were rotating or moving to and fro in a limited space. When a heat filter was imposed movement stopped. When the filter was removed after a latent period of from a fraction of a second to about 5 sec., the flagella were seen in active motion. Often there was no appreciable interval between the beginning of flagellar motion and the motion of the whole organism. This is exactly what would be expected if there were no physical obstacle to the movement of the body. Sometimes there was a distinct lag between the commencement of flagellar activity and movement of the bacillary body. This might be anticipated if the body is closely confined between the cover-slip and the agar when the flagellar activity would have to overcome considerable force before the body could move.

Effect of withdrawal of the heat stimulus. Observations were made on the motility of some of the long coiled filaments which only rotated in the microscope field. Movement ceased as soon as a heat filter was placed in the light beam: when stimulated by some exposure to the heat rays the organism moved actively, but it soon became more sluggish and eventually motion ceased. We selected certain organisms and measured the time occupied in making each single revolution after a heat filter had been interposed for various times (Table 1*a*).

This is probably the first time that a bacillus has been timed for laps in a long distance race, and several interesting points emerge from the figures in Tables 1*a* and 1*b*. A rest of 75 sec. gives exactly the same result as a rest of 180 sec. From this it could be argued that in 75 sec. the organisms had regained their maximum of stored energy. When the period of rest is only a few seconds

Table 1*a*. *Motility of a coiled organism after shielding from radiant heat for various periods*

Duration of rest from heat stimulation (sec.)	Revolutions after renewed stimulus: Number at Maximum speed (2–5 sec.)	Number at Slower speed before stopping	Time of a single revolution at the slower speed (sec.)	Total revolutions before stopping
180	23	0	—	23
2	7	1	13	8
75	22	1	9	23
10	13	1	8	14
2	6	2	7, 12	8
5	9	2	8, 25	11
75	23	1	11	24
1	2	2	12, 26	4
5	11	2	6, 7	13
2	7	0	—	7
1	3	1	13	4
180	24	0	—	24

Table 1*b*. *The relation of the duration of rest from heat stimulation, and subsequent revolutions of motile bacteria. Data from Table* 1*a, cols.* 1 *and* 5

Duration of rest from heat stimulation (sec.)	No. of observations	No. of revolutions before motility ceases	Average
180	2	23, 24	23·5
75	2	23, 24	23·5
10	1	14	14
5	2	11, 13	12
2	2	7, 8	7·5
1	2	4, 4	4

the organism ceases to move after much fewer revolutions and between 1 and 10 sec. the revolutions vary directly with the duration of the rest period.

The recuperative power of these organisms seems to be extraordinarily rapid, for even after a period of rest of only 1 sec. an organism which was apparently incapable of movement is able to resume movement for some 15–20 sec.

In these observations flagellar movement started a fraction of a second before movement of the whole organism. At its fastest this organism made 1 revolution in 2 sec. This seems too slow to throw out mucoid substance. The flagella were ready to move as soon as they received the next stimulus. There was no question of the organism moving first and throwing out the flagella. The flagella moved first.

In the long filamentous forms it is common to find a filament moving with undulations of the body just like a serpent, but such a filament very frequently bends over into a close U-shape and moves forward with the bend of the U in front without any undulations of the limbs, and with rope-like flagella in active movement streaming backwards from inside the bend, as reported also by Kingma Boltjes (1948). In a previous communication instances were given of

movements which seemed impossible by simple undulations of the body (Fleming, 1949).

The movements of the organism may be so sluggish that it takes a coiled filament 30 sec. or more to make a single revolution round the central axis. It is inconceivable to us that this slow movement would be capable of throwing off mucoid threads, which, according to Pijper, constitute the so-called flagella. Moreover, the flagella, even in these slow-moving organisms, can easily be seen by phase contrast to be in very active motion. It is much more believable that the flagella are doing their best to move the organism, but by the physical conditions the movement is hampered and very slow.

Quite commonly flagellar movement can be seen in these slide cultures when the swollen organisms appear to be so hemmed in by their neighbours that they cannot move. The flagella here react to heat rays exactly as the flagella of the organisms which are capable of motion.

The movement of these active flagella attached to non-motile organisms was timed in the same way as the movement of motile organisms (Table 2). The

Table 2. *The reaction to heat of flagella on stationary organisms*

Duration of rest from heat stimulation (sec.)	Latent period before movement of flagella recommenced after heat stimulation (sec.)	Duration of the renewed flagella movement (sec.)
180	1	70
3	7	13
3	8	13
10	—	30
60	About 1	50
180	About 1	75

figures show that after a long interval of rest the latent period was much shorter and the length of time during which the flagella were active was much longer. After being stimulated many times by short periods of rest, the flagella appeared to become completely exhausted and ceased to respond to the heat stimulus. Clearly these active flagella, which can be stopped and started at will by exposure to heat rays, cannot be merely mucoid strands cast off by the active movement of the organism as Pijper maintains. There is no movement to cast them off. It might be held that before the organism became closed in by its neighbours so that it could not move, it had cast off these mucoid strands which still remained attached to the body. If that were so it would be strange if these inanimate strands were capable of responding to heat by going into active motion exactly as though attached to organisms which can move. In these the flagella stop and the organism stops when the heat filter is introduced again. Movement of the flagella and of the organism starts again when the heat filter is removed but the body never moves before the flagella.

It has not been possible to see the flagella by phase contrast where there was fluid between the agar and the cover-slip. In such conditions the motility of the organism was usually greater, but even where it was sluggish the flagella

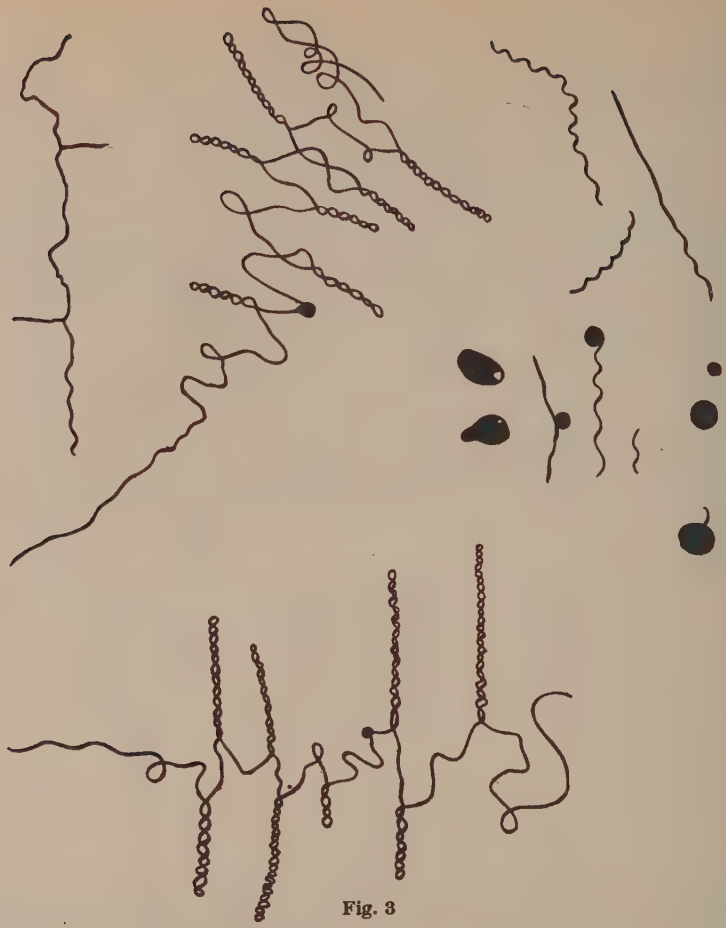


Fig. 3

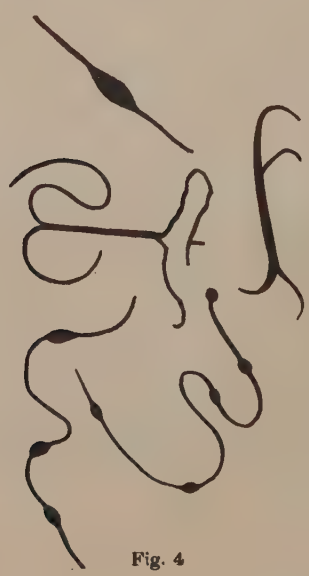


Fig. 4

could not be seen. Doubtless this is because, in these circumstances, the flagella, arising as they do from the whole surface of the organism, are projecting in all directions, when whereas the organism is lying between the agar and the cover-slip they can only be in one plane and so are more readily focused. The movement of the spherical forms is of some interest. We have shown that they possess flagella. The position of the vacuoles permits orientation of the sphere to be determined during the whole period of observation. Even a rolling movement of the body could be excluded; it seemed clear that motility was due to some outside influences and the most likely outside influences were the flagella.

These organisms were large—if movement were due to gyratory undulations of the body then one would expect to see such undulations, but usually the body showed nothing of the sort. The movements are frequently such that it would be impossible for them to take place unless we suppose they are caused by a pushing movement of something outside the body and if we reject Pijper's contention and accept the old idea that the flagella are actually the organs of motility, all the movements can be explained.

We have succeeded in preparing stained specimens in undisturbed slide cultures of *P. vulgaris* by the method indicated earlier. The bacteria were arranged as seen in the living culture; the flagella were clearly visible—spread out where the bacteria were not closely opposed or in wavy 'ropes', where the bacteria were close to each other or where the coiled filaments of the same organisms were closely apposed.

Organisms other than Proteus vulgaris

We have discussed the changes in *P. vulgaris* grown in penicillin. Very similar morphological changes can be observed with *Salmonella typhi* using the same penicillin concentrations. Swellings and protozoon-like forms are common, but more especially there is a predominance of long filamentous forms.

Bacterium coli showed the same changes, but to a lesser degree, with concentrations of 50–500 u./ml. penicillin in the agar slide cultures.

Vibrio cholerae, grown in concentrations of penicillin of 5–20 u./ml., show large numbers of spiral organisms. It appeared as if the organisms grew up to the point at which separation should have taken place, but there the process stopped. Each element had the typical curved form, but instead of being separate curved vibrios they had the spirillum-like appearance. Frequently this was still more exaggerated and single filaments could be seen which had twisted themselves into extraordinary shapes (Fig. 3).

In stronger concentrations of penicillin and especially at 37° all the organisms had grown into bulbous protozoon-like elements resembling those of *P. vulgaris* but smaller. Our results with *Vibrio cholerae* largely confirm those of White (1950).

Pseudomonas pyocyanea was more resistant but in 5000–10,000 u./ml. of penicillin agar long threads and bulbous enlargements occurred. Sometimes branched forms were observed (Fig. 4).

We wish to thank Mr Ben May of Mobile, Alabama, for a research grant to one of us and to Mr May and the American Optical Company for gifts of phase-contrast microscopes which enabled this work to be done, and Dr Cardew of the Photographic Department here for his help and advice.

REFERENCES

Ainley Walker, E. W. & Murray, W. (1904). The effect of certain dyes upon the cultural characters of *Bacillus typhosus* and other micro-organisms. *Brit. med. J.* ii, 16.

Duguid, J. P. (1948). The influence of cultural conditions on the morphology of bacterium aerogenes with reference to nuclear bodies and capsule size. *J. Path. Bact.* **60**, 265.

Fleming, A. (1949). *The Nature of the Bacterial Surface*, p. 166. Edited by A. A. Miles and N. W. Pirie. Oxford: Blackwell's Scientific Publications Ltd.

Gardner, A. D. (1940). Morphological effects of penicillin on bacteria. *Nature, Lond.*, **146**, 837.

Kingma Boltjes, T. Y. (1948). Function and arrangement of bacterial flagella. *J. Path. Bact.* **60**, 275.

Klieneberger-Nobel, E. (1949). Origin, development and significance of L-forms in bacterial cultures. *J. gen. Microbiol.* **3**, 439.

Pijper, A. (1946). Shape and mobility of bacteria. *J. Path. Bact.* **58**, 325.

Robinow, C. F. (1945). Addendum to *The Bacterial Cell*, ed. R. J. Dubos. Cambridge, Mass.: Harvard Univ. Press.

Spray, G. H. & Lodge, B. M. (1943). The effects of resorcinol and of *m*-cresol on the growth of *Bact. lactis aerogenes*. *Trans. Faraday Soc.* **39**, 424.

Webb, M. (1948). The influence of magnesium on cell division. *J. gen. Microbiol.* **2**, 275.

White, P. B. (1950). A note on the globular forms of *V. cholerae*. *J. gen. Microbiol.* **4**, 36.

EXPLANATION OF PLATES

Plate 1

Figs. 1–6. The development of abnormal forms in contact with penicillin. Serial photographs by phase contrast. (×860.)

Fig. 1. A young culture 1½ hr. after seeding on to penicillin agar 5 u./ml. Originally all the organisms were the same size; growth and division of the two lower organisms.

Fig. 2. After 4 hr. Long threads with well-marked nuclear dots.

Fig. 3. After 4½ hr. Swellings appear on the threads and a loop forms at the tip of the right-hand bundle.

Fig. 4. After 5 hr. Part of the right-hand bundle streams down to form a rotating spiral.

Fig. 5. After 6½ hr. Swellings continue to develop. The first-formed spiral has ceased to rotate, and a new one has formed from the upper loop of the left-hand group.

Fig. 6. After 19 hr. Vacuolation well marked. Fragmentation of threads.

Plate 2

Fig. 7. Bacilli in the early stages of penicillin-induced change, stained for nuclei. (×2150.)

Figs. 8, 9. More advanced stages, showing condensation of nuclear material. (×4300.)

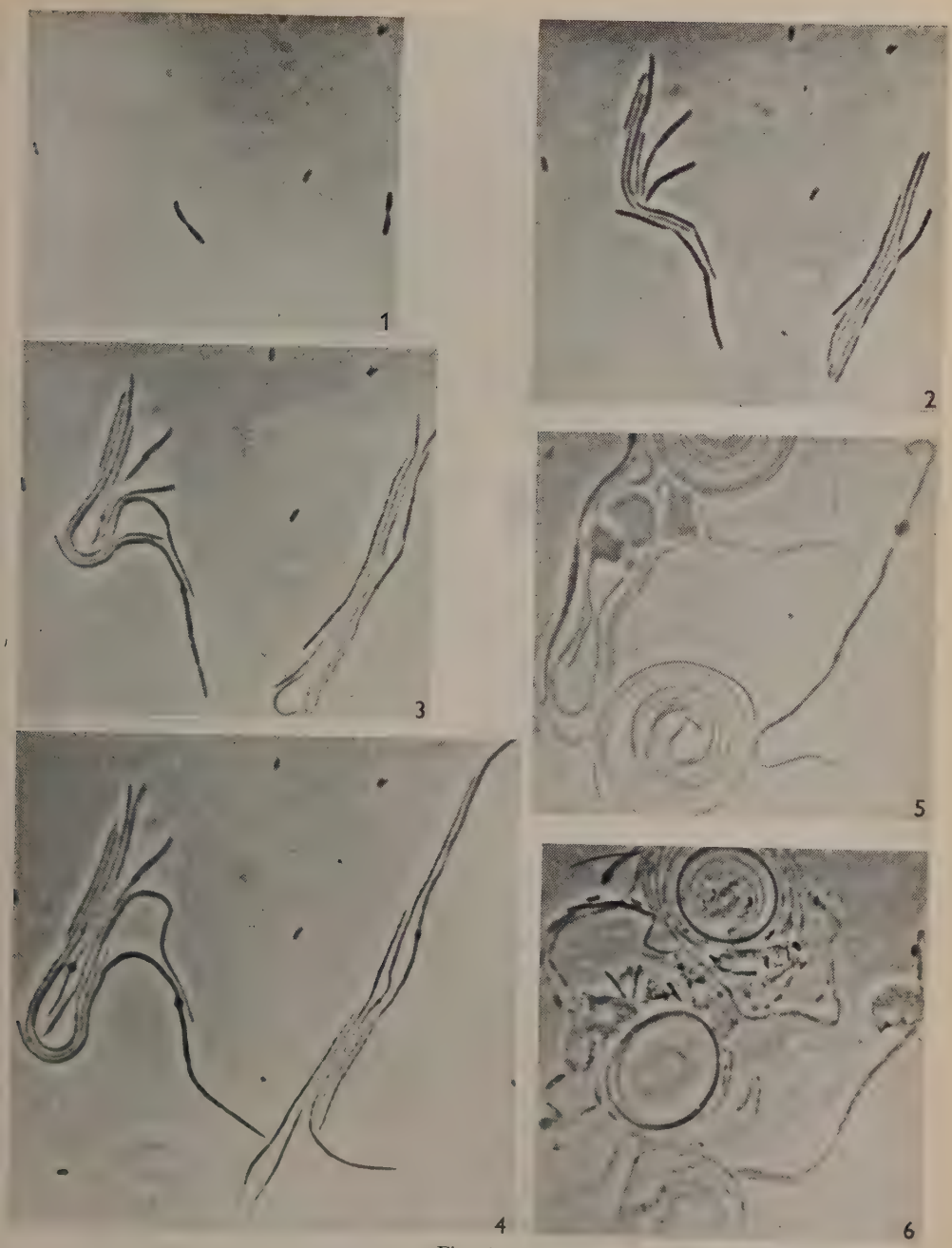
Figs. 10–12. Larger forms showing condensed nuclear material. (×2150.)

Fig. 13. Large swellings. Diffuse nuclear material.

Fig. 14. Large swellings. Central condensation of nuclear material.

Fig. 15. Large swellings. Reticular pattern of nuclear material.

Fig. 16. Very large swelling with central reticular nucleus.

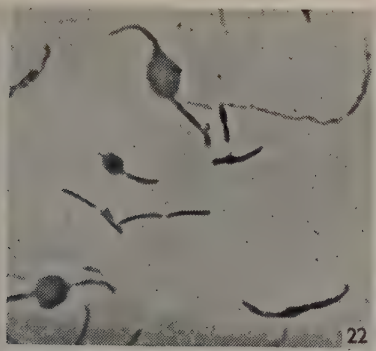


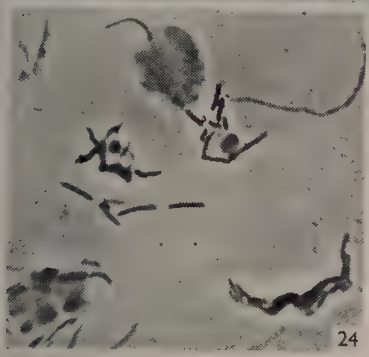
Figs. 1–6

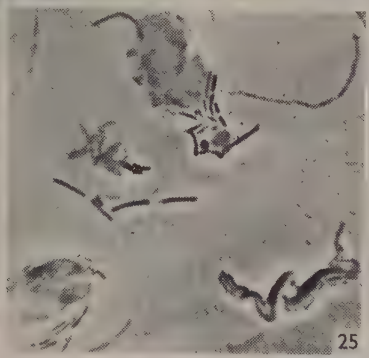
A. Fleming, A. Voureka, I. R. H. Kramer & W. H. Hughes—Penicillin-induced changes in *P. vulgaris*. Plate 1

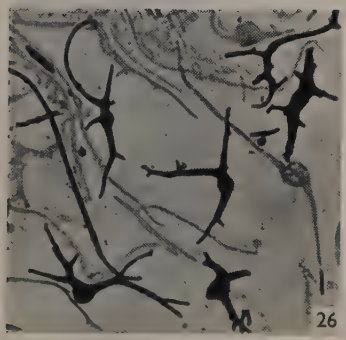
Figs. 7–21

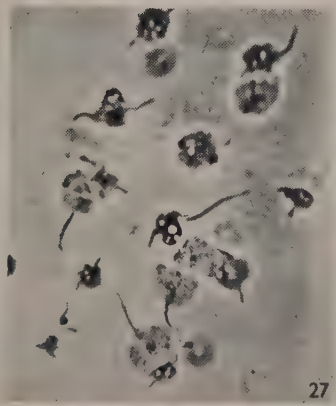
A. Fleming, A. Voureka, I. R. H. Kramer & W. H. Hughes –Penicillin-induced changes in *P. vulgaris*. Plate 2

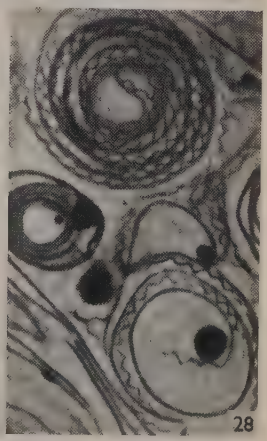












Figs. 22–28

A. Fleming, A. Voureka, I. R. H. Kramer & W. H. Hughes —Penicillin-induced changes in *P. vulgaris*. Plate 3

Fig. 17. Vacuolated swelling, reticular nucleus displaced by vacuoles.

Fig. 18. 'Pool' with threads resting on the edge and spherical forms in the deeper layer of fluid at the centre. (×250.)

Figs. 19–21. Flagella of abnormal forms.

PLATE 3

Figs. 22–25. The degenerate cover-slip culture shown in Pl. 1, fig. 6, was transferred to penicillinase-agar to allow recovery to take place. Serial photographs by phase contrast. (×720.)

Fig. 22. Immediately on transference to penicillinase-agar.

Fig. 23. After 1 hr. Swellings enlarge, the more normal forms begin to divide. Tails of swellings divide.

Fig. 24. Swellings begin to disintegrate.

Fig. 25. Tails and surviving cells continue to divide to give bacilli of normal size. A number of organisms in the field show no sign of recovery though not necessarily as abnormal in appearance as those that resume growth.

Fig. 26. Branched forms and ghosts, 20 u. penicillin/ml. (×720.)

Fig. 27. Field of cells all affected by a high concentration of penicillin, 50 u./ml.

Fig. 28. Stained spirals showing flagella ropes.

(*Received* 21 *October* 1949)

A Growth-Inhibitory Effect on *Shigella dysenteriae* which Occurs with some Batches of Nutrient Agar and is Associated with the Production of Peroxide

By H. PROOM, A. J. WOIWOD, JOAN M. BARNES and W. G. ORBELL

The Wellcome Research Laboratories, Beckenham, Kent

SUMMARY: Certain batches of nutrient agar were inhibitory to small inocula of *Shigella dysenteriae*. The inhibitory effect was abolished by catalase and by manganese dioxide. It occurred only with surface cultures incubated aerobically: poured plates or plates incubated anaerobically showed no inhibition. The inhibitor was a peroxide formed during the heating of nutrient broth and agar; it was present in minimal inhibitory concentrations since dilution with one to four volumes of saline agar removed the inhibition. It is suggested that some nutrient agars contain material which, on exposure to air, forms inhibitory concentrations of peroxide in the surface layer.

During the examination of papain digest broths for their growth-promoting properties one of us (H. P.) made the following observation. *Shigella dysenteriae*, strain CN 191 from the Wellcome Bacterial Collection, was used to test two types of broth—broth (*a*) an undiluted papain digest of horse muscle containing about 7·5 g. total N/l., and broth (*b*) which consisted of broth (*a*) diluted with an equal volume of 0·5 % saline. The smallest inoculum giving growth was the same with both broths. The broths were then converted into nutrient agars by the addition of 2·0 % Japanese agar. The growth from a heavy surface inoculum was about the same with both nutrient agars, but with dilute inocula more colonies grew from the agar made with the diluted broth than on the agar from the undiluted broth. Moreover, progressive dilution of the nutrient agar made from broth (*a*) with saline agar increased the number of colonies growing from dilute inocula. No such difference was observed when silica gel was substituted for agar as the solidifying agent.

The failure of the test organism to grow thus appeared to be due to some inhibitor. The inhibitor was not present in the broth, since the smallest inoculum which grew was the same with both broths. Similarly, the inhibitor was not present in the agar itself, since dilution of a 'bad' nutrient agar with saline agar removed the inhibition. The effect was also observed during the development of tests for standardizing bacteriological media. It was subjected to systematic study and the results are reported below.

EXPERIMENTAL

Preparation of the nutrient agars

Nutrient broth was prepared by extracting fresh minced horse muscle with hot water in the proportion of *c.* 700 g. muscle to 1 l. water. The meat particles were removed by filtration and to the extract was added a concentrated

peptone solution equivalent to an additional total nitrogen content of 1·5 g./l. Then 0·125 % $CaCl_2$ was added and the pH adjusted to 7·8. NaCl was added to a final concentration of 0·5 % and the mixture boiled, filtered and sterilized by Seitz filtration. The total nitrogen content of this broth was about 3·0 g./l. The peptone used was prepared by digesting horse muscle at pH 5·8 with papain for 2 hr. at 60°. The mixture was then boiled and sterilized by filtration. The total nitrogen content of this concentrated peptone was about 14 g./l. Nutrient agar was prepared by adding 1·25 % New Zealand agar (Davis Gelatin (N.Z.) Ltd., Christchurch, New Zealand) to nutrient broth. The nutrient agar was filtered hot and sterilized by autoclaving.

Comparison of growth on different batches of nutrient agar

A convenient test for comparing the growth on different batches of solid medium was the determination of the viable count of a bacterial suspension by the Miles & Misra (1938) technique, using an arbitrarily selected batch of medium as a standard for comparison. An overnight culture on nutrient agar of strain CN 191 was emulsified in Ringer's solution and diluted to an opacity corresponding to Brown's opacity tube No. 3 (Burroughs, Wellcome Ltd.), i.e. approximately 2000×10^6 organisms/ml. Single drops, from a 0·02 ml. dropping pipette, of ten-fold serial dilutions of this suspension were dropped on agar plates and incubated in the usual manner. Four dilutions were used for each plate.

The difference between good and bad nutrient agars

Although all batches of nutrient agar used were prepared by the same standard method, two types were encountered. A 'good' nutrient agar gave confluent growth with an inoculum of a dilution of 10^{-5} of the test suspension, about 50 colonies at a dilution of 10^{-6}, and an occasional colony at 10^{-7}. On the other hand, a 'bad' nutrient agar gave confluent growth with a dilution of 10^{-1} and no growth with the subsequent dilutions. The two types of nutrient agar were indistinguishable when tested against *Escherichia coli* or *Staphylococcus aureus*, the bad nutrient agar having no inhibitory action against these organisms. This effect was shown to be a species effect and was not confined to the particular strain of *Sh. dysenteriae* used. Six other strains of *Sh. dysenteriae* were inhibited by the bad nutrient agar but no strains of the species *Sh. paradysenteriae*, *Esch. coli*, or *Staph. aureus* were affected.

Dilution of broth with saline before the preparation of nutrient agar or the dilution of a bad nutrient agar with saline agar eliminated the inhibitory effect. A typical protocol is given in Table 1, showing that relatively slight (1/3) dilution with saline agar was sufficient to remove the inhibition. Dilution with one or two volumes of saline agar was always sufficient to overcome any inhibitory effect.

Effect of various substances on the inhibition

A systematic search was made for substances which might neutralize the inhibitory effect of bad nutrient agars. A large number of growth factors, separately or together, casein acid-hydrolysate, starch, or thiolacetic acid

Table 1. *The effect of dilution of good and bad nutrient agars with saline agar on the growth of* Shigella dysenteriae *from small inocula*

Medium	Growth from 0·02 ml. inoculum of dilution						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Good agar (batch M 259/48)	+	+	+	+	+	25	3
Good agar (3 vol.) + saline agar (1 vol.)	+	+	+	+	+	22	2
Good agar (1 vol.) + saline agar (1 vol.)	+	+	+	+	+	28	1
Good agar (1 vol.) + saline agar (3 vol.)	+	+	+	+	+	46	6
Bad agar (batch M 319/48)	+	—	—	—	—	—	—
Bad agar (3 vol.) + saline agar (1 vol.)	+	—	—	—	—	—	—
Bad agar (1 vol.) + saline agar (1 vol.)	+	+	+	96	8	—	—
Bad agar (1 vol.) + saline agar (3 vol.)	+	+	+	+	+	26	3

Undiluted inoculum = *c.* 2×10^9 organisms/ml. + = confluent growth or numerous colonies. Numerals = no. of colonies.

Table 2. *Neutralization of the inhibitory effect of a bad nutrient agar on* Shigella dysenteriae *by dilutions of purified catalase*

Medium	Growth from 0·02 ml. inoculum of dilution			
	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Good agar (batch M 259/48)	+	+	37	3
Bad agar (batch M 840/48)	—	—	—	—
15 ml. bad agar + 1 ml. 0·85 % saline	—	—	—	—
15 ml. bad agar + 1 ml. catalase solution diluted 10^{-2}	+	+	48	6
15 ml. bad agar + 1 ml. catalase solution diluted 10^{-4}	+	+	51	9
15 ml. bad agar + 1 ml. catalase solution diluted 10^{-6}	+	+	19	—
15 ml. bad agar + 1 ml. catalase solution diluted 10^{-8}	+	88	8	—
15 ml. bad agar + 1 ml. catalase solution diluted 10^{-10}	+	17	3	—

Undiluted inoculum = *c.* 2×10^9 organisms/ml. + = confluent growth or numerous colonies. Numerals = no. of colonies.

(0·01 % incorporated in the medium) did not neutralize the inhibitor. The inhibitory effect was, however, abolished by fresh blood, some vegetable extracts such as extract of horse radish, a purified catalase preparation and manganese dioxide, but not by salts of manganese. With the exception of manganese dioxide, the ability of these substances to neutralize the inhibitor was destroyed by heat. Table 2 shows the neutralization of the inhibitory effect by purified catalase (kindly supplied by Dr D. Herbert).

The abolition of the inhibition by high dilutions of catalase, but not by heated catalase, and also by manganese dioxide, but not by salts of manganese, can be regarded as reasonable proof that the inhibition was due to a peroxide. This agrees with the observation that catalase-producing organisms such as *Sh. paradysenteriae*, *Esch. coli*, or *Staph. aureus* were not inhibited by bad nutrient agars; *Sh. dysenteriae* does not produce catalase.

Manganese dioxide destroyed the inhibitory effect of a bad nutrient agar. This was shown either by incorporating a little solid MnO_2 in the medium (Table 3) or by placing a little MnO_2 on the surface of an agar plate. In the latter case, when the surface of a plate was inoculated with a dilute inoculum,

colonies of *Sh. dysenteriae* grew in the immediate vicinity of the MnO_2 particles but not elsewhere. Table 3 also shows that when a bad nutrient agar containing MnO_2 was filtered the filtrate was no longer inhibitory. However, when this filtered agar was autoclaved it again became inhibitory. This indicated that the inhibitor or its precursor was formed, or its formation was rapidly accelerated, during the heating of this nutrient agar.

Table 3. *The effect of* MnO_2 *on the inhibitory action of a bad nutrient agar on* Shigella dysenteriae

Medium	Growth of *Sh. dysenteriae* with 0·02 ml. inoculum of dilution			
	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Good agar (batch 259/48)	+	130	13	1
Good agar + MnO_2 (1 g./l.)	+	210	14	4
Bad agar (batch 840/48)	—	—	—	—
Bad agar + 1 g./l. MnO_2	+	200	16	3
Bad agar + 1 g./l. MnO_2 and filtered*	+	79	12	1
Bad agar + 1 g./l. MnO_2 filtered and autoclaved†	6	—	—	—
Bad agar + 0·001 % $MnSO_4$	—	—	—	—

* Filtered through Seitz pads. † Autoclaved 15 min. at 15 lb.

Undiluted inoculum = *c.* 2×10^9 organisms/ml. + = confluent growth or numerous colonies. Numerals = no. of colonies.

It was possible to convert a good nutrient agar into a bad nutrient agar by continuous autoclaving. The degree of autoclaving necessary to do this varied from batch to batch. But here it was necessary to distinguish between a bad nutrient agar of the type under investigation and nutrient agars which by continued heating had lost growth-promoting properties for other species. This was done by noting whether the inhibition was removed by catalase and whether the nutrient agar was still capable of growing *Staph. aureus*.

Attempts made to extract the inhibitor were uniformly unsuccessful. Saline extracts of bad agars were made and tested in parallel with nutrient broth. The smallest inoculum to grow was the same in both cases. When these extracts were reconverted into nutrient agar by steaming with 1·25 % agar they were non-inhibitory. When the dilutions of test inoculum were made in extracts from bad nutrient agars and tested on a good nutrient agar no inhibition was observed. These results would confirm that the peroxide was present only in minimal inhibitory amounts. Chemical tests for peroxide in bad nutrient agars were negative.

The smallest inocula to grow on good and bad nutrient agars were compared by the poured plate method as well as by the surface method of Miles & Misra. No inhibition was shown with a bad nutrient agar as a poured plate. Good and bad nutrient agars were then compared when surface-inoculated plates were incubated anaerobically; under anaerobic conditions a bad nutrient agar was no longer inhibitory. Sandwich plates were made with various combinations of good and bad nutrient agars. Table 4 illustrates the results obtained. The

first layer was poured, dried and inoculated, and after the inoculum had dried the second layer of agar was then poured on top. When the two layers were both of bad nutrient agar some inhibition was obtained, but with all other combinations little inhibition was observed.

Table 4. *The effect of sandwich plates and anaerobiosis on the growth of* Shigella dysenteriae *on good and bad nutrient agars*

Medium	Growth of *Sh. dysenteriae* with inoculum of dilution			
	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Good agar (batch M 402/48) incubated aerobically	+	+	19	6
Bad agar (batch M 605/58) incubated aerobically	—	—	—	—
Good agar (M 402/48) incubated anaerobically	+	+	25	1
Bad agar (M 605/48) incubated anaerobically	+	+	19	3
Sandwich plates: good agar upon bad agar	+	+	18	2
Sandwich plates: bad agar upon good agar	+	31	9	1
Sandwich plates: bad agar upon bad agar	19	20	4	—
Sandwich plates: good agar upon good agar	+	+	21	2

Undiluted inoculum = *c.* 2×10^9 organisms/ml. + = confluent growth or numerous colonies. Numerals = no. of colonies.

The surface of some bad agar plates was removed by careful scraping or by slicing off the top layer, and then inoculated on the new surface by the usual technique. Inhibition was again observed, thus showing either that the inhibition was not confined to the surface layer or that a new inhibitor was formed on exposure of the fresh surface to air.

Attempts were made to simulate bad nutrient agar by the addition of hydrogen peroxide to good nutrient agar. In a number of experiments whenever an inhibitory concentration of peroxide was present in the medium, growth on both surface-inoculated and poured plates was inhibited. It was not found possible to produce only surface inhibition by addition of hydrogen peroxide.

DISCUSSION

The complex nature of nutrient agar and the small amount of peroxide that must be present makes the identification of the inhibitor, or of the mechanism leading to its formation, extremely difficult. The removal of the inhibition by catalase or manganese dioxide may be accepted as reasonable proof that the inhibitor is indeed a peroxide. This peroxide is present in amounts which cannot be detected by the usual chemical tests. Since dilution with one to four volumes of saline agar usually abolishes the inhibition, the peroxide present must be near the limit of its minimal bacteriostatic concentration. Various authors have shown that exposure of nutrient media to sunlight, ultraviolet radiation or X-rays may produce an inhibitory medium. Burnet (1925) demonstrated that peroxide was formed in nutrient agar exposed to sunlight. Wyss, Clark, Haas & Stone (1948) showed that organic peroxides

were formed in broth irradiated with ultraviolet light. Blank & Arnold (1935) found that irradiated carbohydrates or agar were inhibitory to *Bacillus subtilis* when incorporated into nutrient agar. These results differ in several respects from those described here. In the experiments of Burnet (1925) and Wyss *et al.* (1948) the concentration of peroxide formed was sufficient to inhibit a catalase-producing organism such as *Staph. aureus*, whereas in our experiments the concentration of peroxide formed was only sufficient to inhibit a non-catalase-producing organism. However, on one occasion, prolonged heating of a batch of bad nutrient agar produced a very dark medium which inhibited *Staph. aureus* and *Sh. dysenteriae* with both surface and poured plate cultures. This inhibition was overcome by catalase. In this respect our results may be regarded as quantitatively different from those quoted. Another difference is the mechanism by which peroxide is formed. In our experiments irradiation was not a significant factor. The peroxide was formed by reaction between material present in some broths and material normally present in agar. The production of peroxide was associated with heat and the presence of atmospheric oxygen. In fact, it was found possible to produce a bad agar by bubbling oxygen through melted good nutrient agar maintained at 47° in a water bath. In this case the agar became lighter in colour, demonstrating that the production of peroxide is not necessarily associated with the darkening of nutrient agar which occurs on prolonged heating. On the available evidence it seems that when some nutrient broths are heated with agar-agar some material is formed which on exposure to atmospheric oxygen readily produces an inhibitory concentration of peroxide. This material is distributed throughout the medium, but the peroxide is apparently confined to the surface layer. It is unlikely that peroxide is present throughout the medium since, when inhibitory concentrations of peroxide are added to good nutrient agar, it is inhibitory when inoculated by either the pour-plate or surface technique. Moreover, it may seem unlikely that peroxide would not be inhibitory to *Sh. dysenteriae* when growing under microaerophilic (or anaerobic) conditions. However, it is not possible to dismiss entirely the possibility that these low concentrations of peroxide may be distributed uniformly throughout the medium and that only the strictly aerobic metabolism of *Sh. dysenteriae* is affected.

These results are of some practical interest in connexion with the preparation of nutrient agars, particularly for surface growth from small inocula. They also have a more general appeal because of the interest which is being shown in the study of growth inhibitors and the conditions under which they arise. Peroxide is another example of such an inhibitor and the destruction of peroxide may be another function of the blood used in some bacteriological media. Since peroxide formation is always a possibility when the surface of organic material is exposed to the atmosphere the production of catalase will be an advantage to aerobic bacteria.

REFERENCES

Blank, H. I. & Arnold, W. (1935). The inhibition of growth of *Bacillus subtilis* by ultraviolet irradiated carbohydrates. *J. Bact.* **33**, 507.

Burnet, F. M. (1925). Hydrogen peroxides and bacterial growth. *Aust. J. exp. Biol. med. Sci.* **2**, 77.

Miles, A. A. & Misra, S. S. (1938). Estimation of bactericidal power of blood. *J. Hyg., Camb.*, **38**, 732.

Wyss, O., Clark, J. B., Haas, F. & Stone, W. S. (1948). The role of peroxide in the biological effects of irradiated broth. *J. Bact.* **56**, 51.

(*Received* 2 *November* 1949)

The Utilization of Amino-Acid Solutions by Virus-Infected Eggs, Studied by Paper Chromatography

By M. van den ENDE
The Department of Pathology, University of Cape Town

SUMMARY: The rate of utilization of amino-acids injected into the allantoic cavity of virus-infected eggs was studied by paper-strip chromatography.

Influenza virus brought about more rapid disappearance of amino-acids from the allantoic fluid than Mumps, Vaccinia or Lumpy Skin viruses or diphtheria and *Clostridium welchii* toxins. The utilization of amino-acids appears to be non-specific and not directly concerned with virus synthesis.

Increasing attention is being paid to the chemical processes in virus multiplication. In particular the bacteriophage has been studied (cf. Cohen, 1949). Spizizen (1943) described the effect of certain amino-acids on the release of bacteriophage from infected host cells suspended in simple media. Animal viruses have, however, received relatively little attention, though Bauer (1948) has reported an increase in xanthine oxidase in virus-infected chick embryos and mouse brain.

Paper chromatography (Consden, Gordon & Martin, 1944) appeared to us to be well suited for studying the utilization of amino-acids and other substrates by virus-infected tissues, and in particular by eggs, which are generally used for the cultivation of animal viruses.

We undertook this work in the hope of discovering differences between the normal and virus-infected eggs which might elucidate the intracellular synthesis of viruses.

METHODS

Solutions of amino-acids were prepared containing four to six amino-acids which would separate well on simple strip-paper chromatograms with phenol or butanol as solvent. The solutions were made in distilled water, the concentration of each amino-acid being 0·015 M, adjusted to a pH of *c.* 7, Seitz filtered and stored in the dark in small screw-capped bottles.

Eggs incubated for 9 days received 0·2 ml. of virus suspension or saline into the allantoic cavity, and were held at 35°. After 1½–18 hr. the eggs were opened through a triangular flap over the burr-hole of the inoculation, and the allantoic cavity opened without allowing the chorio-allantoic membrane to drop. The allantoic fluid was pipetted off with sterile precautions and replaced by 2·0 ml. of amino-acid solution or saline. The triangular opening was closed with cellophan tape. After further incubation for 15–72 hr. each egg was chilled in the ice chest, reopened by cutting away the cellophan window, and the allantoic fluid was collected. The allantoic cavity was then washed with sterile saline and the washings pooled with the original fluid to make a total volume of 10 ml. Each pool was tested for sterility and, where practicable, titrated for virus content.

For strip chromatograms single $1\frac{1}{4}$ in. strips of Whatman No. 1 filter paper were used. Two volumes of 100 μl. of fluid from a single egg were then applied to each strip; the second volume after the first had completely dried. A large museum jar and a shallow glass dish made a convenient apparatus to hold up to 16 strips at a time. Temperature was not controlled, but strips with known concentrations of the original amino-acid solution were included in each jar for comparison of colour intensities after development with ninhydrin. Convenient separation was usually obtained in 10–12 hr. at room temperature, after which the strips were dried at 80° and developed with ninhydrin. In some experiments, filter-paper sheets were substituted for single strips and butanol was used as solvent.

The virus strains used were Influenza A (PR 8 and Lepine), Swine influenza, Mumps (Enders strain), Lumpy Skin Disease and Vaccinia. In addition, diphtheria and *Clostridium welchii* toxins were tested to determine whether non-specific factors were responsible for the effects observed with viruses.

The dosage of virus or toxin had to be adjusted so that the embryo did not die during the experiment, because post-mortem autolysis resulted in the rapid appearance of amino-acids which obscured those introduced.

RESULTS

Virus titres of the pools collected were almost invariably low when allantoic fluid was replaced by saline 12 hr. or more after infection of the eggs.

The results of typical experiments using one amino-acid mixture are summarized in Table 1. The disappearance of amino-acids from the allantoic cavity of eggs infected with influenza virus is rapid compared with that from control eggs receiving saline instead of virus inoculum, or from eggs infected by other viruses. All the six amino-acids are equally affected and there is no evidence of any selective utilization. Similar results were obtained with mixtures of (*a*) methionine, hydroxyproline, threonine, serine and valine; and (*b*) glycine, lysine, leucine and proline. When the period of incubation between virus inoculation and allantoic fluid replacement was 18 hr. and the second period of incubation was shortened to 15–18 hr. the rapid disappearance of the amino-acids was still demonstrable, but when the interval between infection with influenza virus and the introduction of amino-acids was decreased to $1\frac{1}{2}$ hr. the accelerated disappearance of the amino-acids in the subsequent 24 hr. was no longer striking. This suggests that the effect is non-specific, possibly due to the inflammatory reaction set up by the influenza virus. But the fact that influenza virus infection differed so strikingly from infection by other viruses that are also capable of multiplication in the cells lining the allantoic cavity, and from inflammation produced by toxins in the allantoic fluid, remains unexplained. It is possible that the other infective agents and the toxins under the particular experimental conditions produced a less severe inflammation or that the inflammation was accompanied by different metabolic derangements of the host cells. Such metabolic derangements may be the direct result of the virus infection.

Material giving positive reaction with ninhydrin occurred in normal as well as in virus-infected eggs, but no attempt was made to identify it.

Table 1

Inoculum	Allantoic fluids replaced by	Incubation after replacement (hr.)	Final contents of allantoic cavity. Colour intensity of amino-acids on chromatogram*						Virus haemaglutinin titre
			Aspartic acid	Glutamic acid	Glycine	Alanine	Histidine	Phenylalanine	
Influenza A	Amino-acids	48	—	—	—	Tr.	—	+	1/160
	Amino-acids	48	Tr.	—	Tr.	—	—	+	1/320
	Saline	48	—	—	—	—	—	—	1/320
Saline	Amino-acids	48	+	+	+	+	+	+	—
	Amino-acids	48	+	+	+	+	++	+	—
Lumpy skin	Amino-acids	48	+	+	+	+	+	+	†
	Amino-acids	48	+	+	+	+	+	+	†
Saline	Amino-acids	48	++	++	++	++	++	++	—
Swine flu	Amino-acids	48	Tr.	—	—	+	Tr.	—	1/40
	Amino-acids	48	Tr.	—	—	+	—	—	1/20
Saline	Amino-acids	48	+++	+++	+++	+++	+++	+++	—
Swine flu	Saline	48	—	—	—	—	—	—	1/20
Mumps	Amino-acids	72	+	+	+	+	+	+	1/160
	Amino-acids	72	+	+	+	+	+	+	1/160
Saline	Amino-acids	72	+	+	+	+	+	+	—
	Amino-acids	72	++	++	++	++	++	++	—
Mumps	Saline	72	—	—	—	—	—	—	1/160
Diphtheria toxin‡	Amino-acids	48	++	++	+	++	+	+	—
Saline	Amino-acids	48	+	++	++	++	+	+	—

* Tr. = trace of colour. +, ++, +++ = colour intensities corresponding respectively to one-eighth, one-sixth and one-quarter of that given by the original solution of amino-acids.

† Fluids produced specific death in 100 % of eggs in 3–4 days.

‡ Dose = 0·5 minimal lethal dose for eggs.

This work was carried out with a grant from the South African Council of Scientific and Industrial Research. Dr F. M. Burnet kindly supplied the strain of Mumps virus used. We are grateful to Dr T. S. Work for some of the chromatograms.

REFERENCES

Bauer, D. J. (1948). Association of xanthine oxidase with virus multiplication. *Nature, Lond.*, **161**, 852.

Cohen, S. S. (1949). Growth requirements of bacterial viruses. *Bact. Rev.* **13**, 1.

Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). Qualitative analysis of proteins: a partition chromatographic method using paper. *Biochem. J.* **38**, 224.

Spizizen, J. (1943). Biochemical studies on the phenomenon of virus reproduction. I. Amino-acids and the multiplication of bacteriophage. *J. infect. Dis.* **73**, 212.

(*Received 7 November* 1949)

Jensenia, A New Genus of the Actinomycetales

By K. A. BISSET and F. W. MOORE
Bacteriology Department, University of Birmingham

It has been brought to our attention that in the absence of a type species the new genus *Jensenia* proposed in our recent paper (Bisset & Moore, 1949) is invalid according to the international rules of bacteriological nomenclature (Buchanan, St John-Brooks & Breed, 1948, 1949). We therefore now formally propose a new genus *Jensenia* (Actinomycetales, Actinomycetaceae) based on the new species *Jensenia canicruria*.

Jensenia Bisset & Moore, n.g.

Cells short, often irregular in shape, occasionally branched. Aerobic.

Type species *Jensenia canicruria* Bisset & Moore.

Jensenia canicruria Bisset & Moore, n.sp.

Rods and very short filaments containing one to four or five cells, each 1·0–1·2 by 5–10 μ. Angular forms common. Occasional, short, impermanent branches. Non-sporing, non-capsulated. Outline irregular.

Non-motile. Gram-positive, non-acid-fast.

Obligate aerobe. Optimum temperature 30–33°.

Grows slowly on routine media; growth apparent after 48 hr.

Surface colonies circular, white, waxy, emulsifiable in saline. Forms a pellicle on fluid media. Does not ferment carbohydrates nor hydrolyse polysaccharides or proteins. Does not reduce nitrates. Capable of utilizing pyridine and allied cyclic compounds as sole carbon, nitrogen and energy source.

Isolated from soil.

Subcultures of the type culture have been deposited at the National Collection of Type Cultures, Colindale, London, N.W. 9.

REFERENCES

Bisset, K. A. & Moore, F. W. (1949). The relationship of certain branched bacterial genera. *J. gen. Microbiol.* 3, 387.

Buchanan, R. E., St John-Brooks & Breed, R. S. (1948). International bacteriological code of nomenclature. *J. Bact.* 55, 287. Ibid. (1949). *J. gen. Microbiol.* 3, 444.

(*Received* 11 *November* 1949)

No. 2, Volume 4 of the Journal of General Microbiology was issued on 11 May 1950

A New Aquatic Nitrogen-Fixing Bacterium from Three Cambridgeshire Chalk Streams

By E. GRAY and J. D. SMITH

The School of Botany and the Molteno Institute, University of Cambridge

SUMMARY: A motile rod-shaped bacterium which on nitrogen-free media fixed appreciable quantities of nitrogen gas was isolated in pure culture from three chalk streams near Cambridge. It was weakly Gram-positive and beaded in stained preparations. Flagellation was distinctive and unusual in that the flagella at the poles were coarser than those at the sides. Growth was promoted by sugars, and nitrate was rapidly reduced. Since the flagellation of the organism is unusual, its classification is difficult; but the organism does not appear to have been previously described.

During an investigation of the bacteria of a chalk stream, Hobson's Brook, near Cambridge, a nitrogen-fixing organism was isolated in April 1948, and 2 days later from a mill-pool (Byron's Pool) on the River Cam and about a quarter of a mile away from a second small stream, the Bourn Brook, which empties into Byron's Pool. At that time it was most abundant in Hobson's Brook. The organism was not found in any of these streams during the summer, and no further search was made for it in the Cam or the Bourn Brook. In October 1948 it was again found in some abundance in Hobson's Brook, but not during the winter months. It reappeared, again in some quantity, in Hobson's Brook in the spring of 1949. In Hobson's Brook it was found in greatest abundance in the spring and autumn at certain points where green plants persist all the year round, but this may have no significance.

The organism could not be identified, but as it was thought possible that it might be one of the soil nitrogen-fixing bacteria, a description of it was sent to the Rothamsted Experimental Station. A reply was received stating that it was not one of the known soil forms, and it was suggested that a subculture be sent to Prof. A. J. Kluyver who is at present examining it. The organism has still not been identified. Micrographs taken with the electron microscope by Dr A. L. Houwink have shown that it possesses a most unusual kind of flagellation, which has added further difficulties to the determination of its systematic position.

The three streams from which the organism was first isolated are all alkaline in reaction (pH 7·4–7·6). Hobson's Brook and one tributary of the Cam above Byron's Pool flow from chalk springs, while the Bourn Brook is derived from surface-water, some of which must originate from the chalk underlying this region of the Cambridge district. All three streams are in the country 3 miles from Cambridge, and flow through arable and old grass-land. They are, however, sharply distinguished by their degrees of turbulence. The Bourn Brook is sluggish, about 8 ft. wide and 6 ft. deep where the organism was isolated, and flows between banks of soft earth. The Cam enters Byron's Pool over a weir,

while immediately below the springs the half-mile of Hobson's Brook from which the organism was isolated is swift, shallow and subject to great changes in the volume of water. The bed of this half-mile of Hobson's Brook is composed of alternate stretches of chalk and gravel. Owing to an origin from springs and the swift current, the temperature of Hobson's Brook is lower but more uniform (averaging 10° all the year round) than that of the Cam or the Bourn Brook, which range between 8° in winter and 15° in summer. The amount of dissolved oxygen in Hobson's Brook in the half-mile below the springs is nearly always near saturation; in the Cam and the Bourn Brook it is probably much less owing to the quantity of suspended organic matter.

In Hobson's Brook the organism was found only near three areas where green plants (notably *Apium nodiflorum*, *Veronica anagallis* and *Nasturtium officinale*) exist all the year round. Some unidentified filamentous green algae floated near the point where the organism was found in the Bourn Brook (clear water *c.* 4 ft. from bank). No green plants or algae were present where the organism was taken from the Cam (clear water *c.* 4 ft. from bank).

Method of sampling. Samples were taken from the length of Hobson's Brook and at Byron's Pool and the Bourn Brook, in sterile 8 oz. 'medical flat' bottles having rubber stoppers covered by a cap of Kraft paper. Mannitol (3·0 g.) and K_2HPO_4 (0·2 g.) were added to each bottle before sampling. Samples were taken directly by hand in the current core of Hobson's Brook. The samples from the Bourn Brook and from Byron's Pool were taken from clear water away from banks, by securing the bottles to the end of a rod. The bottles were filled to capacity, restoppered, covered by the Kraft paper cap, and well shaken to dissolve the contents. The temperature of the stream was then taken, and the pH immediately determined colorimetrically on a second sample. On returning to the laboratory, the sample bottles were again well shaken and half their contents poured into sterile plugged 250 ml. conical flasks. The remainders of the samples were left in the bottles, whose mouths were plugged with cotton-wool.

Method of isolation

The flasks and bottles were allowed to stand in the shade at *c.* 15°. After 4–6 days a waxy pellicle appeared on the surface of three samples taken from those points where green plants were found all the year round in Hobson's Brook but not from elsewhere in the Brook, and also on the surface of the samples taken from the Cam and the Bourn Brook. Platinum loopfuls of this pellicle were streaked over the surface of mannitol soil agar medium, and azotobacter (mannitol phosphate agar) medium, and incubated at 22°. The azotobacter medium contained 0·04 g. K_2HPO_4, 0·02 g. KH_2PO_4, 1·5 g. mannitol, with *c.* 0·1 g. each of NaCl, $CaCO_3$ and garden soil, with 150 ml. distilled water and 2·5 g. washed agar to form a solid medium. After 2 days white opaque pin-point colonies appeared on both media. In one sample from Hobson's Brook, heaped butyrous semi-translucent colonies also appeared on the mannitol soil agar but not on the azotobacter medium. These colonies were subcultured on mannitol soil agar until pure cultures were obtained. The larger

butyrous colonies were those of an organism identified by Dr E. G. Pringsheim as a species of Monad, a small colourless flagellate. The small white colonies were those of an organism whose description follows.

The organism

Morphology. A motile rod about 6–8 μ. long. Weakly Gram-positive, showing a beaded appearance suggestive of a diphtheroid. Non-acid-fast when first isolated. Stained by Morton's night blue method, the flagella were peritrichous. Electron micrographs by Dr A. L. Houwink (Delft) showed that the organism had most unusual flagella in that those at the poles appeared coarser than those at the sides. Dr Houwink regards this particular flagellation as remarkable and quite distinctive.

Growth on various media. Although in one instance isolated in association with a colourless flagellate the organism was subsequently maintained alone in pure culture. On all solid media a good growth appeared in 4 days. On mannitol soil agar slopes the colonies were small, thick, white and moist, becoming confluent; on azotobacter medium growth was rather poor, the colonies being opaque, gelatinous and discrete. Similar scattered colonies appeared on plain milk agar and on Yeastrel milk agar. Growth was most profuse on media containing sugar or glycerol. On slopes of Yeastrel glucose agar a moist, diffuse, grey growth with a thick white deposit in the water of condensation appeared. On Yeastrel glycerol agar a similar growth was obtained but white, not grey, in colour. On potato plugs a moist, sticky, confluent growth with a yellow colour was obtained. Gelatin stab cultures (gelatin in Lab-Lemco broth) were not liquefied after 6 weeks, but the medium turned pink. In bouillon (made with Lab-Lemco broth) a faint turbidity with a granular deposit appeared very slowly over 6 weeks. Growth in litmus milk (made with milk powder) was very poor and there was no visible change in the medium after 6 weeks.

Biochemical reactions. Acid but not gas was produced in 2 weeks in peptone-free sugar media containing lactose, maltose, glucose, or sucrose. Salicin was not attacked in 6 weeks. Indole was not produced. The Voges-Proskauer and methyl red reactions were negative; litmus milk was not changed; methylene blue was not reduced. Nitrate was rapidly reduced to nitrite, but ammonia was not formed.

Metabolism. Aerobic, no growth in stab cultures except at surface. Psychrophilic, growing best between 10° and 20°. Pigment formed. Growth greatly improved by sugar and glycerol. Resistance to heat not determined. Isolated from alkaline waters and grew well on media at pH of 7·2–7·4. Growth at other pH values and the calcium requirements were not examined.

Fixation of nitrogen gas. Shortly after isolation the micro-organism was found able to grow in the absence from the medium of combined nitrogen. As the growth observed in media to which no nitrogen compound had been added might have been due to traces of nitrogenous impurities, some preliminary quantitative measurements of the nitrogen gain by growing cultures were made. The nitrogen content of cultures was measured before and after growth by the

micro-Kjeldahl technique (Markham, 1942). The medium used had the following composition: K_2HPO_4, 0·8 g.; KH_2PO_4, 0·2 g.; NaCl, 0·2 g.; $MgSO_4.7H_2O$, 0·2 g.; $CaSO_4.2H_2O$, 0·1 g.; $Fe_2(SO_4)_3.9H_2O$, 0·01 g.; mannitol, 10 g.; distilled water to 1 l. (pH 7·3). Sterile 100 ml. conical flasks each containing 10 or 15 ml. of medium were inoculated with 0·1 ml. of a suspension of the bacteria grown on an agar slope of the same medium. Samples (10 ml.) from the flasks were analysed for total nitrogen immediately after inoculation, similar samples being taken from other flasks after incubation at 20° for several days. Correction was made for any changes in the volume of the contents of the flasks during incubation. The purity of the cultures was examined microscopically at the conclusion of each experiment.

The changes in nitrogen content of these cultures are shown in Table 1. All cultures showed a small increase in nitrogen content which was well outside the limits of experimental error. It was concluded that the micro-organism absorbed small quantities of atmospheric nitrogen (probably limited in these experiments by the rate of diffusion of gas into the medium). The measurements reported are only preliminary ones.

Table 1. *Nitrogen content of samples* (10 *ml.*) *of cultures incubated at* 20°

	Incubation period	Nitrogen in sample (mg./10 ml. culture)	Nitrogen gained (mg./10 ml. culture)
Exp. 1	Zero (control)	0·036	—
	Zero (control)	0·040	—
	4 days	0·057	0·019
	4 days	0·068	0·030
Exp. 2	Initial sample	0·010	—
	Initial sample	0·010	—
	3 days	0·019	0·009
	3 days	0·023	0·013
	3 days	0·032	0·022
Exp. 3 (medium with 0·1 % yeast extract added)	Initial sample	0·049	—
	3 days	0·068	0·019
	3 days	0·057	0·009
	3 days	0·055	0·006

Spectroscopic examination. Thick suspensions of the organism were examined by the microspectroscope for evidence of the presence of haematin compounds. On reduction with sodium dithionite the absorption bands of the cytochromes were immediately visible. These appeared to be identical with those observed in suspensions of various strains of *Rhizobium*. The α bands of cytochromes *a*, *b* and *c* could be distinguished; those of cytochromes *b* and *c* were extremely close together but were easily resolved on cooling to liquid air temperature.

We wish to thank Dr A. L. Houwink for permission to reproduce the electron micrographs.

Fig. 1

Fig. 2

E. Gray & J. D. Smith—A new aquatic nitrogen-fixing bacterium from three Cambridgeshire chalk streams. Plate 1

REFERENCES

MARKHAM, R. (1942). A steam distillation apparatus suitable for micro-Kjeldahl analysis. *Biochem. J.* **36**, 790.

EXPLANATION OF PLATE

Figs. 1 and 2. Micro-photographs of the organism taken with the electron microscope by Dr A. L. Houwink. The distinctive flagellation, coarser at the poles than at the sides is shown

(*Received* 15 *October* 1949)

Anaerobic and Aerobic Growth of Purple Bacteria (Athiorhodaceae) in Chemically Defined Media

By S. H. HUTNER

Haskins Laboratories, New York 17, *N.Y.*

SUMMARY: Studies on the anaerobic growth of aerobically adapted purple bacteria show that the same growth factors suffice for both aerobic and anaerobic growth, thus supporting van Niel's formulations of photosynthesis and carbon assimilation in these forms, and his thesis that the previously observed need for peptone or yeast extract could be attributed to their content of essential growth factors. Combinations of glutamate and succinate (or fumarate) were especially good promoters of growth in malate media; it is possible that their effectiveness was referable to their allowing a by-pass of the CO_2 requirement. The production of molecular hydrogen by purple bacteria is discussed in relation to the reducing intensities attained in cultures.

A bottle technique for anaerobic cultures, and a flask technique for aerobic cultures, are described in detail.

Photosynthesis in purple bacteria is best observed when they are grown anaerobically (van Niel, 1944, 1949). It was therefore important to determine whether the growth factors, aneurin, nicotinic acid, biotin and *p*-aminobenzoic acid, needed for aerobic growth (Hutner, 1946), would suffice for anaerobic growth. The experiments described here indicate that although the culture media had to be modified to conform to certain special requirements imposed by anaerobic growth, the required growth factors remained the same.

The need for rigorous anaerobiosis when comparing anaerobic with aerobic growth is illustrated by *Staphylococcus aureus*, which requires to be given uracil only when kept strictly anaerobic (Richardson, 1936). Another cautionary example of a difference of growth-factor requirements as between aerobic and anaerobic conditions is provided by certain lactobacilli which require vitamin B_{12}; anaerobically vitamin B_{12} is replaceable for them with substances which are completely inactive aerobically (Welch & Wilson, 1949).

Investigations of this type call for the elimination of fortuitous traces of organic impurities; furthermore the confusions introduced by inapparent but growth-factor-producing microbial contamination of stock solutions have also to be eliminated. The methods here described proved adequate for the purpose and suitable for the study of photosynthetic forms: e.g. the vitamin B_{12} requirement of the algal flagellate *Euglena gracilis* (Hutner, Provasoli, Stokstad, Hoffmann, Belt, Franklin & Jukes, 1949) was identified by these methods. A detailed account of these procedures may contribute to the reproducibility of experiments involving exceedingly active and ubiquitous growth factors.

EXPERIMENTAL METHODS

Anaerobic technique

The anaerobic bottle technique of van Niel (1931, 1936) served as basis. The culture vessels were '20 ml.' Pyrex bottles with interchangeable stoppers. The

capacity of some bottles was 25 ml., the average being *c.* 24 ml.; for convenience the bottles were assumed to have a volume of 20 ml. In practice, bottles each containing 20 ml. of culture media were autoclaved with 50 ml. beakers serving as temporary covers. The glass stoppers were sterilized separately in Petri dishes. The bottles were inoculated. A reducing agent, usually 2 % (w/v) Na formaldehyde-sulphoxylate (Na F-S), was pipetted in to give a final concentration of 0·05 %. The bottles were filled to the necks with distilled water. The necks were filled with a 1:1 mixture of petrolatum and heavy paraffin oil, and sealing of the bottles was completed by replacing the covering beakers with sterile glass stoppers. The cultures were then incubated at room temperature (22–30°) under tungsten lamps. Growth was practically complete in 2 weeks with vigorous cultures; some required a month or longer. Growth was estimated visually.

Reducing agents. Na F-S was satisfactory. Its reducing intensity approached the limit set by the potential of the hydrogen electrode, as demonstrated by the ability of low concentrations (0·02–0·04 %) in culture media to reduce completely benzylviologen ($E_0' - 0{\cdot}359$ V.; Michaelis & Hill, 1933) at pH 7. That Na F-S induced lower potentials than thiolacetate, cysteine or ascorbate was noted by Reed & Orr (1943). Sulphide was less satisfactory because of the narrow range between the effective reducing and toxic concentrations, and it precipitated some of the heavy metals in the medium. Na dithionite (hydrosulphite; $Na_2S_2O_4 . 2H_2O$) proved too unstable in solution for consistent results. The following were non-toxic but lacked adequate reducing intensity: 1-thioglycerol (Evans Research and Development Corp., New York City), acetaldehyde Na bisulphite and Na benzenesulphinate. Na thiolacetate and mercaptoethanol were inhibitory. The oxidation-reduction system Na F-S and oxygen has not been investigated chemically. A sample of Na F-S consisting of pieces *c.* 1 cm. in diameter, stored in air, kept the same reducing efficiency over a period of $3\frac{1}{2}$ years as determined by the rough measure of anaerobic experiments.

Redox indicators. Roelofsen (1935) cultivated purple bacteria in darkness in peptone media at *c.* $-0{\cdot}29$ V., the potential rising in light to $-0{\cdot}19$ V. The cultures also produced H_2 in the dark. The indigosulphonates (La Motte Chemical Products Co., Towson, Maryland) and benzylviologen (British Drug Houses Ltd.) were therefore used as redox indicators (Hewitt, 1948). The indigosulphonates were not toxic, but there were indications that benzylviologen was inhibitory at concentrations above 0·6 mg./100 ml. at pH 7·0. Since benzylviologen is a quaternary base resembling the bactericidal quaternary ammonium compounds, it should be less inhibitory in media more acid than those used here. The Na indigosulphonates were generally used at a concentration of 1·0 mg./100 ml. and benzylviologen at 0·5 mg./100 ml. In the reduced state benzylviologen is blue and the indigosulphonates are colourless. When media were coloured by these dyes there was definite fading after illumination for a few days, and the colour was usually completely discharged in 2–3 weeks. Aerobic or anaerobic cultures kept in the dark did not show serious fading for the duration of an experiment. This fading was rather a help; the initial full

colour facilitated detection of insufficient anaerobiosis, and the subsequent fading simplified estimation of growth. In instances where the dyes were presumably in the colourless state rather than faded at the completion of experiments their presence was tested for, using hypochlorite for the indigo-sulphonates and dithionite for benzylviologen. As reactive dye invariably proved still to be present these tests were eventually discontinued. The use of high concentrations of indicator brought the advantage of a greater poising of the media; this was not noticeably advantageous when appreciable amounts of yeast extract or peptone were present, but the dyes made a significant contribution to the poising of simpler chemically defined media. In work with substrates it should be borne in mind that reduced redox indicators may serve as H-donators (van Niel, 1931). Indigodisulphonate is readily available commercially as 'indigocarmine'.

Notes

(1) Culture bottles are available on request with a sand-blasted spot for labelling.

(2) To minimize auto-oxidation the reducing solution was prepared just before use, dispensed in deep screw-capped tubes plugged with Pyrex glass-wool (non-irritating to the respiratory tract), and the tubes quickly chilled after autoclaving. Distilled water for filling the bottles to volume was dispensed from flasks similarly fitted with glass-wool plugs, and likewise autoclaved and chilled just before use. These precautions were necessary to avoid consumption of excessive and variable amounts of reducing agent in order to reach the desired reducing intensities.

(3) When larger bottles were used the changes in fluid volume attendant upon sterilization were such that the addition of relatively large amounts of distilled water was needed. With the 20 ml. bottles water was conveniently dispensed by means of cut-down 100 ml. volumetric pipettes. These pipettes were sterilized by steam in glass tubes plugged at both ends with cotton-wool.

(4) The petrolatum-paraffin oil mixture ('vaspar') was dispensed from screw-capped bottles. For sterilization these bottles were heated in a metal desiccator for 2 hr. at 180–190°. To avoid entrainment of dust on cooling, the desiccator lid was sealed with Dow-Corning silicone stopcock grease. The vaspar was heated gently before pouring.

Aerobic technique and general methods

Aerobic cultures used for controls were maintained in 25 ml. Erlenmeyer flasks capped with beakers, and containing 10 ml. of medium. Pyrex flasks having a short neck (the new model) were provided with glass caps 22 mm. high and 22 mm. in diameter.

All stock solutions were preserved with a mixture of (v/v) 1 part *o*-fluorotoluene, 2 parts *n*-butyl chloride, and 1 part 1:2-dichloroethane (Hutner & Bjerknes, 1948). This preservative volatilized on autoclaving. *o*-Fluorotoluene is obtainable in the United States from Eastman Kodak Ltd.

Media and glass stoppers were autoclaved 25–40 min. at 118–121°. To avoid intake of dust on cooling, the exhaust valve of the autoclave was kept shut for at least 5 hr. after the period of sterilization. If the autoclave chamber still maintained a degree of vacuum at this time, it was brought to atmospheric pressure very gradually before opening. Petri dishes containing glass stoppers were placed in Pyrex kitchenware trays ($12\frac{5}{8} \times 8\frac{1}{8} \times 2$ in. $= 32 \times 20{\cdot}6 \times 5$ cm.),

as were the culture bottles and flasks. Upon removal from the autoclave the trays were covered with another inverted tray. Such double-tray containers were especially useful with the aerobic beaker-capped flask cultures. A tray of flasks could be moved about with little risk of contamination, and the double trays containing bottles or flasks could be stacked, conserving space, and, the units being transparent, conserving light. Each double-tray unit was in function the counterpart of a giant Petri dish accommodating twenty-eight 25 ml. flasks or eighteen 50 ml. flasks. For added rigidity and protection from dust the joint between the upper and lower trays was sealed with transparent cellulose tape.

Culture media

As the present work centred on the requirement for growth factors rather than on carbon or energy metabolism, it was permissible to use DL-malate throughout the experiments as metal-carrier (Hutner, 1948), hydrogen donator, and potential source of CO_2. This last function depended on malate being more oxidized than cell material; therefore its complete dissimilation should lead to a net production of CO_2 (Muller, 1933). Malate alone seldom permitted good anaerobic growth. Addition of glutamate, especially in combination with fumarate or succinate, allowed excellent growth; this clear-cut superiority was not evident in the aerobic controls. Good media devoid of materials of biological origin, or devoid of nitrogen except for the negligible amount contained in the growth factors, could be devised by replacing the glutamate with substrates especially suited for particular strains. Acetate and butyrate were generally useful for this purpose; glycerol was especially well utilized by

Table 1. *Basal medium for identification of growth-factor requirements under anaerobic conditions*

	g.		mg.
K_2HPO_4	0·05	Na indigodisulphonate	1·0, or
$MgSO_4.7H_2O$	0·025	Benzylviologen	0·5
DL-Malic acid	0·3	Zn	1·0
Na_2 succinate.$6H_2O$	0·4	Ca	0·4
L-Glutamic acid	0·2	Mn	0·2
Glycerol	0·2	Fe	0·1
K acetate	0·1	Cu	0·1
		Mo	0·05
		Co	0·05

Distilled water to 100 ml.

pH adjusted to 6·8–7·1 with KOH.

Na formaldehyde sulphoxylate 0·05 g./100 ml. added separately as a freshly autoclaved 2 % solution.

Growth factors supplied when necessary as follows: aneurin 0·1 mg., nicotinic acid 0·1 mg., *p*-aminobenzoic acid 0·01 mg., and biotin 0·4 μg.

The concentrations of metals listed refer to the metal content of the salt used. These were usually the sulphates.

Rhodopseudomonas palustris. Experiments along these lines showed that all strains could use ammonium ion as principal nitrogen source. A representative medium which allowed good growth of strains of all five species studied, at a reduction level where benzylviologen developed nearly full colour and indigo-

disulphonate was completely decolorized (the potential thus nearing that of the hydrogen electrode) is shown in Table 1.

Inoculations were carried out by suspending growth from slants of 'synthetic' agar media (made up with minimal amounts of growth factors) in the growth-factor free basal medium in aerobic flasks, and inoculating each bottle with a drop of the suspension. These dilution flasks therefore provided a check on the magnitude of carry-over effects. In other respects the procedures were those already described (Hutner, 1946).

RESULTS

The growth factor requirements for anaerobic growth appeared identical with those found for aerobic growth. Of four isolates of *R. capsulatus*, three showed an absolute requirement for aneurin, with nicotinic acid and biotin further increasing growth; a fourth strain which grew irregularly aerobically also grew irregularly anaerobically. The four isolates tested of *R. gelatinosa* grew in aneurin + biotin. Three isolates of *R. spheroides* grew in aneurin + biotin + nicotinic acid. Nine out of ten isolates of *R. palustris* required *p*-aminobenzoic acid; the other strain grew poorly both aerobically and anaerobically. Seven out of nine strains of *Rhodospirillum rubrum* required biotin; the other two strains grew poorly.

A typical comparison of anaerobic and aerobic growth is that given for *R. rubrum* (Table 2). The medium was similar to that in Table 1 except that acetate was omitted. It is noteworthy that strain 6, which is the 'S 1' strain used by Gest & Kamen (1949*a*, *b*), was one of the two strains which did not grow well in these media. The other species gave similar impressions of strain difference and of a generally faster and denser aerobic growth.

In many experiments strains grown in darkness (necessarily under aerobic conditions) showed the usual growth factor requirements.

Table 2. *Comparison of anaerobic and aerobic growth of* Rhodospirillum rubrum

(pH 7·0. Anaerobiosis was achieved by the addition of 0·04 % Na formaldehyde sulphoxylate. Duration of experiment: 30 days.)

Strain:	1	2	5	6	7	10	15	17	20
				Anaerobic					
Relative amounts of growth									
−Biotin	0	0	0	0	±	0	0	±	0
+Biotin	+	++	+	0	0	++	++	+++	++
				Aerobic					
−Biotin	0	±	±	±	±	±	±	±	±
+Biotin	+++	++++	±	±	++++	+++	±	++++	±

DISCUSSION

The present findings support van Niel's thesis that the indispensability for purple bacteria of yeast extract or peptone resided in their content of essential growth factors, and that the peptone or yeast extract in his media did not significantly affect the stoichiometric relations postulated for their photosynthetic and assimilatory activities. The superior growth-promoting power of the glutamate-succinate mixtures (succinate was preferred because of its synthetic origin) pointed to a special function for these compounds. That this may be the by-passing of the CO_2 requirement for initiation of growth is suggested by the work of Lwoff & Monod (1947) who found that glutamate or succinate, preferably in combination, allowed growth of *Escherichia coli* in CO_2-free air with a medium already containing glucose. Glutarate and asparagine were also effective, but fumarate, acetate, pyruvate, and lactate were inactive. These experiments were extended by Ajl & Werkman (1949) to the anaerobic growth of *Esch. coli* and *Aerobacter aerogenes*: here α-ketoglutarate, glutamate, glutamine, and oxaloacetate were the best replacements for free CO_2. However, for the germination of spores of *Clostridium botulinum* glutamate was ineffective in replacing CO_2, but a mixture of L-malate, fumarate and succinate was partially effective; oxaloacetate was completely effective, and aspartate inactive (Wynne & Foster, 1948). In the present experiments no effort was made to deprive the cultures of CO_2 nor was carbonate added; a cultivation technique permitting thorough removal of CO_2 should help in elucidating the nature of the glutamate-fumarate stimulation in purple bacteria.

The isolates studied in the present investigation generally grew faster in the less reduced media. This apparent preference must be interpreted with caution as all these strains were adapted to aerobic conditions. The technique employed here allowed exposure of the inoculum to air, and hence would be ill-advised for the obligately anaerobic *Rhodospirillum fulvum* (Giesberger, 1947).

Certain species of purple bacteria are brown when grown anaerobically and red when grown aerobically (van Niel, 1947). This phenomenon furnished an independent way of detecting reducing conditions, but the colour shift occurred at a potential range higher than that used for the growth-factor studies.

The observations of Gest & Kamen (1949*a*, *b*) add theoretical interest to the assessment of the likelihood that certain strains of purple bacteria may develop very low oxidation-reduction potentials. They found that a strain of *R. rubrum* produced molecular hydrogen vigorously when grown with aspartate or glutamate as nitrogen source, and with malate, fumarate or succinate as additional substrates. In agreement with the results reported here, they were able to grow their strain on a chemically defined medium containing biotin, with NH_4Cl as principal nitrogen source. A determination of the potential limits for the production of hydrogen might provide an elegant method for judging the degree of anaerobiosis of cultures; should the potentials approach the region of hydrogen overvoltage some of the evolved H_2 might have originated from H_2O rather than from the substrate. A review of the present anaerobic bottle experiments favours the possibility that a similar evolution of H_2 occurs in

other species of purple bacteria, particularly among strains of *Rhodopseudomonas palustris* and *R. capsulatus*: certain strains were prone to form gas bubbles, while other strains, growing to the same extent, did not develop bubbles. Furthermore, this gas formation was not correlated with the level of oxidation of the substrate—an indication that a gas other than CO_2 was being formed. The yeast extract found helpful for vigorous growth by Gest & Kamen (1949*b*) appeared unnecessary when heavy metals are supplied as in the medium listed in Table 1.

This investigation was aided by a grant from the American Philosophical Society.

REFERENCES

Ajl, S. J. & Werkman, C. H. (1949). Anaerobic replacement of carbon dioxide. *Proc. Soc. exp. Biol., N.Y.*, **70**, 522.

Gest, H. & Kamen, M. D. (1949*a*). Photoproduction of molecular hydrogen by *Rhodospirillum rubrum*. *Science*, **109**, 558.

Gest, H. & Kamen, M. D. (1949*b*). Studies on the metabolism of photosynthetic bacteria. IV. Photochemical production of molecular hydrogen by growing cultures of photosynthetic bacteria. *J. Bact.* **58**, 239.

Giesberger, G. (1947). Some observations on the culture, physiology and morphology of some brown-red *Rhodospirillum* species. *Antonie v. Leeuwenhoek J. Microbiol. Serol.* **13**, 135.

Hewitt, L. F. (1948). *Oxidation-reduction potentials in bacteriology and biochemistry*, 5th ed. London: Staples Press Ltd. (Agents, London County Council.)

Hutner, S. H. (1946). Organic growth essentials of the aerobic nonsulfur photosynthetic bacteria. *J. Bact.* **52**, 213.

Hutner, S. H. (1948). Essentiality of constituents of sea water for growth of a marine diatom. *Trans. N.Y. Acad. Sci.* Ser. II, **10**, 136.

Hutner, S. H. & Bjerknes, Clara A. (1948). Volatile preservatives for culture media. *Proc. Soc. exp. Biol., N.Y.*, **67**, 281.

Hutner, S. H., Provasoli, L., Stokstad, E. L. R., Hoffmann, C. E., Belt, M., Franklin, A. L. & Jukes, T. H. (1949). Assay of anti-pernicious anemia factor with *Euglena*. *Proc. Soc. exp. Biol., N.Y.*, **70**, 118.

Lwoff, A. & Monod, J. (1947). Essai d'analyse du rôle de l'anhydride carbonique dans la croissance microbienne. *Ann. Inst. Pasteur*, **73**, 323.

Michaelis, L. & Hill, E. S. (1933). The viologen indicators. *J. gen. Physiol.* **16**, 859.

Muller, F. M. (1933). On the metabolism of the purple sulphur bacteria in organic media. *Arch. Mikrobiol.* **4**, 131.

Niel, C. B. van (1931). On the morphology and physiology of the purple and green sulphur bacteria. *Arch. Mikrobiol.* **3**, 1.

Niel, C. B. van (1936). On the metabolism of the Thiorhodaceae. *Arch. Mikrobiol.* **7**, 32.

Niel, C. B. van (1944). The culture, general physiology, morphology, and classification of the non-sulfur purple and brown bacteria. *Bact. Rev.* **8**, 1.

Niel, C. B. van (1947). Studies on the pigments of the purple bacteria. III. The yellow and red pigments of *Rhodopseudomonas spheroides*. *Antonie v. Leeuwenhoek J. Microbiol. Serol.* **12**, 156.

Niel, C. B. van (1949). The comparative biochemistry of photosynthesis. In *Photosynthesis in plants*, p. 437, ed. J. Frank & W. E. Loomis. Ames, Iowa: Iowa State College Press.

Reed, G. B. & Orr, J. H. (1943). Cultivation of anaerobes and oxidation-reduction potentials. *J. Bact.* **45**, 309.

Richardson, G. M. (1936). The nutrition of *Staphylococcus aureus*. Necessity for uracil in anaerobic growth. *Biochem. J.* **30**, 2184.

Roelofsen, P. A. (1935). On the metabolism of the purple sulphur bacteria. *Proc. Acad. Sci. Amst.* **37**, 660.

Welch, A. D. & Wilson, M. F. (1949). Mechanism of the growth-promoting effect of ascorbic acid on *Lactobacillus leichmanii* and the reduction of oxidation products of vitamin B_{12}. *Arch. Biochem.* **22**, 486.

Wynne, E. S. & Foster, J. K. (1948). Physiological studies on spore germination, with special reference to *Clostridium botulinum*. III. Carbon dioxide and germination, with a note on carbon dioxide and aerobic spores. *J. Bact.* **55**, 331.

(*Received* 18 *October* 1949)

The Serological Identity of a Yellow-Pigmented *Streptococcus*

By C. L. HANNAY

National Institute for Research in Dairying, University of Reading

SUMMARY: A yellow-pigmented *Streptococcus* was isolated from certain dairy cows. The three strains examined were closely related to *Strep. faecalis* but differed from it in fermenting raffinose. The organism produced a tyrosine decarboxylase. Serologically the organism belonged to Lancefield group D, Sharpe type 10.

The literature contains many references to streptococci which produce pigment under anaerobic, or semi-anaerobic conditions, for example, in deep agar or broth cultures (Durand & Giraud, 1923; Lancefield, 1934; Plummer, 1941). The *Streptococcus* reported here produced pigment on the surface of aerobic plates.

It was isolated three times from the teats of one cow which was being used in an experiment at the Institute by my colleague, Mr F. K. Neave, on the prevention of the spread of *Strep. agalactiae* by means of nisin. It was not isolated from any of sixty other cows in the Institute herd. It was also isolated twice from the hands of the milker concerned. When first noticed it was in almost pure culture on the plate and may have been overlooked earlier. A year later a similar organism was isolated from another herd in which no antibiotics had been used.

Three strains, two isolated from the Institute's herd and one from the other herd, have been identified biochemically and serologically as belonging to group D.

SEROLOGICAL METHODS

Preparation of antiserum. Group D sera were produced from the pigmented *Streptococcus* no. 1, *Strep. bovis* 'Rosalie 20', *Strep. faecalis* and from *Strep. durans* (no. 98D). The organisms were grown for 48 hr. at 37° in 1 % glucose Lemco broth, and the suspension prepared in a magnetic shaker according to the method of Shattock (1949). Potent group sera were produced by giving each rabbit, over a period of 3 weeks, six intravenous injections of 1 ml. of freshly prepared suspension.

Precipitin test. Hydrochloric acid extracts (Lancefield, 1933) were layered over serum contained in glass tubes of 3 mm. internal diameter. The juncture of the two fluids was examined after 5 min. for the presence of a precipitate.

Absorption. The complete absorption of group antibodies from potent group D serum appears to require a heavier suspension of organisms than do sera against some of the other Lancefield groups. The technique of Shattock (1949) was therefore used.

RESULTS

General characteristics. On first isolation on blood plates the colonies were strongly pigmented, varying in colour from yellow to dirty brown. On subsequent plating the colonies became very pale but when the growth was aggregated with a platinum loop the bacterial mass was a primrose colour. Stab cultures in nutrient agar were incubated for 24 hr., and when placed in the refrigerator formed a dark yellow brown growth around the site of inoculation. The packed bacterial mass obtained by centrifuging the growth from 24 hr. cultures grown in broth containing 0·2 % dextrose was yellow, and when the supernatant was decanted and the bacterial mass left exposed to the air the pigment was slowly oxidized to a brownish colour.

Biochemical characteristics. The biochemical characteristics of all three strains were identical and conformed to those of *Strep. faecalis*, except that they fermented raffinose. They produced tyrosine decarboxylase (Sharpe, 1948), grew at pH 9·6 (Shattock & Hirsch, 1947), and hydrolysed arginine (Niven, Smiley & Sherman, 1942).

Serology

Serological grouping. This organism presented no difficulties in the production either of a strong extract or of a potent group serum. Extracts reacted with the group D sera produced from *Strep. bovis*, *Strep. durans* and *Strep. faecalis*, but not with the other sera in Lancefield groups A–N. The serum produced from strain no. 1 reacted with extracts prepared from fourteen known group D strains including a number of extracts of *Strep. bovis*, but not with extracts from strains of Lancefield groups A–N or with an extract containing staphylococcal nucleoprotein. Tables 1 and 2 show that strain no. 1 absorbed all group antibodies from the *Strep. durans* and *Strep. bovis* sera and vice versa with serum prepared from strain no. 1.

Table 1. *Absorption of group D antibodies by the pigmented* Streptococcus *no.* 1

	Group D serum			
	Serum '98 D'		Serum 'Rosalie 20'	
Extract	Unabsorbed	Absorbed	Unabsorbed	Absorbed
Strep. durans 98 D	+	−	+	−
Strep. bovis 'Rosalie 20'	+	−	+	−
Strep. faecalis 'C and G'	+	−	+	−
'Pigmented streptococci' (nos. 1–3)	+	−	+	−

Serological typing. The three strains of pigmented bacteria were typed by the slide agglutination and precipitin ring tests, using specific absorbed serum. Each of the three strains reacted specifically with type 10 antiserum (Sharpe, 1949), and gave no cross-reactions with the other twenty type sera available in this laboratory at that time.

DISCUSSION

But for the fermentation of raffinose these chromogenic strains are closely related to *Strep. faecalis*. In this laboratory group D organisms differing from the accepted definition of *Strep. faecalis* are usually regarded as variants, but in the light of the recent work of Sharpe (1949) it seems doubtful whether any

Table 2. *Absorption of group serum from the pigmented* Streptococcus *by group D streptococci*

	Serum 'Pigmented Streptococcus' no. 1		
		Absorbed by	
Extract	Unabsorbed	98 D	'Rosalie 20'
'Pigmented streptococci' (nos. 1–3)	+	–	–
Strep. durans 98 D	+	–	–
Strep. bovis 'Rosalie 20'	+	–	–
Strep. faecalis 'C and G'	+	–	–

raffinose fermenters should be included in the species *Strep. faecalis*. Sharpe tested a large number of strains of group D streptococci isolated from infants' faeces for their tyrosine decarboxylase activity and found that all those organisms which were typical members of *Strep. faecalis* or the varieties *zymogenes* and *liquefaciens*, as well as *Strep. durans*, possessed a high decarboxylase activity, whereas all of those organisms fermenting raffinose, for example *Strep. bovis* and organisms biochemically similar to our chromogenic strains, had little or no tyrosine decarboxylase activity.

The serological type 10 of Sharpe (1949) is rare and has only been identified twice. Neither strain produced pigment or decarboxylated tyrosine. It would appear reasonable to classify the chromogenic strains which fermented raffinose and produced a tyrosine decarboxylase as Lancefield group D, Sharpe type 10, without attempting to give them a specific name.

I should like to express my thanks to Dr A. T. R. Mattick for his interest in these organisms, to Mr F. K. Neave for the cultures, and to Miss M. E. Sharpe for typing the cultures and for permission to refer to unpublished work.

REFERENCES

Durand, P. & Giraud, P. (1923). Les streptococoques chromogènes. *C.R. Acad. Sci., Paris*, **177**, 1333.

Lancefield R. C. (1933). A serological differentiation of human and other groups of haemolytic streptococci. *J. exp. Med.* **57**, 571.

Lancefield, R. C. (1934). Loss of properties of haemolysin and pigment formation without change in immunological specificity in a strain of *Streptococcus haemolyticus*. *J. exp. Med.* **59**, 459.

Niven, C. F., Smiley, K. L. & Sherman, J. M. (1942). The hydrolysis of arginine by streptococci. *J. Bact.* **43**, 651.

Plummer, H. (1941). A serological and biochemical study of haemolytic streptococci. *J. Immunol.* **42**, 91.

SHARPE, M. E. (1948). Some biochemical characteristics of group D streptococci isolated from infants' faeces, with special reference to their tyrosine decarboxylase activity. *Proc. Soc. Appl. Bact.* p. 13.

SHARPE, M. E. (1949). Personal communication.

SHATTOCK, P. M. F. (1949). The streptococci of group D; the serological grouping of *Streptococcus bovis* and observations on serologically refractory Group D strains. *J. gen. Microbiol.* **3**, 80.

SHATTOCK, P. M. F. & HIRSCH, A. (1947). A liquid medium buffered at pH 9·6 for the differentiation of *Streptococcus faecalis* from *Streptococcus lactis*. *J. Path. Bact.* **59**, 495.

(*Received* 19 *October* 1949)

A Study of Phage-Resistant Mutants of *Rhizobium trifolii*

By J. KLECZKOWSKA

Soil Microbiology Department, Rothamsted Experimental Station, Harpenden, Hertfordshire

SUMMARY: Phage-resistant mutants were obtained from four strains of *Rhizobium trifolii*. Mutation to phage resistance tended to coincide with mutations in other features, such as morphology of colonies or effectiveness in nitrogen fixation. The accompanying mutations occurred independently, and their frequency varied widely from strain to strain.

Some mutants seemed stable in their newly acquired features; others continued to mutate at high rates.

Some of the mutants as regards nitrogen fixation were stable or could eventually be stabilized, but other mutants derived from one of the parent strains remained unstable even after several successive replatings or passages through nodules.

Strains of *Rhizobium* belonging to the same inoculation group, i.e. capable of forming root nodules on the same species of leguminous plant, can differ from one another in various respects, such as colonial morphology, serological specificity, effectiveness of nitrogen fixation in root nodules, and ability to ferment different carbohydrates. Individual strains are usually stable, maintaining these features unaltered during many years of cultivation on artificial media. Under ordinary conditions of laboratory cultivation variants with new properties appear only rarely and irregularly. Much work has been done to find conditions in which variants can be obtained more frequently and regularly. Thus Nobbe & Hiltner (1893) and Frank (1899) found that prolonged cultivation on gelatin could lead to a decreased effectiveness in nitrogen fixation; Simon (1908), Hutchinson (1924) and Snieszko (1929) found that the lost effectiveness could be restored by cultivation in soil. On the other hand, by prolonged storage in soil Nutman (1946) obtained ineffective variants from effective strains, but not vice versa.

Allen & Baldwin (1931) obtained more effective variants from less effective strains and vice versa by passages through host plants, but their results did not agree with those of Stapp (1929), and could not be reproduced by Virtanen (1945) or Nutman (1946).

The disagreements between different workers show that the treatments which they described do not regularly give the same results. The divergencies can be explained by assuming that different strains produce mutants which differ in their response to the selective action of a given treatment.

The morphology of colonies of *Rhizobium* strains is another feature whose changes were studied. Almon & Baldwin (1933) obtained variants whose colonies differed from those of parent strains in various respects, some showing yellow or pink pigmentation. Various methods, including treatment with bacteriophage, were used to obtain such variants.

Treatment with a bacteriophage is a rapid means of isolating mutants that are resistant to it. There is evidence that bacterial strains produce phage-

resistant mutants independently of the action of the phage, which merely reveals their presence by destroying susceptible organisms (Burnet, 1929; Luria & Delbrück, 1943; Kleczkowska, 1945*b*; Newcombe, 1949). Fresh mutants will probably be unstable in more respects than one, and so mutations in other features can be expected, at least occasionally, to accompany mutations in susceptibility to a phage. They may include mutations in morphology of colonies (Almon & Baldwin, 1933), and mutations in ability and efficiency to form nodules (Krasil'nikov, 1941).

The purpose of the present work was to study the mutations in colonial morphology and in effectiveness of nitrogen fixation that accompany mutations in phage susceptibility, and to see whether they tend to occur together.

MATERIALS AND METHODS

The four strains of *Rh. trifolii* used were:

Strain A, effective in nitrogen fixation.

Strain B, an ineffective variant obtained by Nutman from strain A by storage in soil and designated as *f* 12 (Nutman, 1946). Strains A and B were identical serologically.

Strains C and D (= strains C and HKC of Nutman, 1946) were both ineffective nitrogen fixers. They were serologically unrelated to each other and to strains A and B.

All the four strains formed greyish white opaque colonies of smooth type, those of strain C being more slimy than the others. The four strains maintained these features unaltered during several years' cultivation on laboratory media and after several passages through host plants.

Two different bacteriophages, both isolated from the soil and maintained in liquid culture as previously described (Kleczkowska, 1945*a*), were used. Strains A and B were treated with phage S_2P_{11} and strains C and D with phage Cl_{15}. Phage S_2P_{11}, which formed large-sized plaques, could lyse strains A and B but not C and D, whereas phage Cl_{15}, which formed small-sized plaques, could lyse all four strains. Phage Cl_{15} was not used with all four strains because the work was started with strains A and B and phage S_2P_{11} when phage Cl_{15} was not available.

The agar medium used for cultivating the bacterial strains, the nitrogen-deficient 'seedling agar' used for growing clover plants in sterile conditions in test tubes, and the technique of growing the plants and of re-isolating bacteria from root nodules, were as described by Nutman (1946).

Late flowering Montgomery red clover was used as host plant. The plants were infected by bacterial strains under test by inoculating the surface of the 'seedling agar' at the time when the first true leaf was opened. Each tested strain was inoculated into a number of tubes each containing two plants. The plants were allowed to grow for about 3 months until eight true leaves were formed on control plants infected with a known strain fully effective in nitrogen fixation. A strain was classified as 'effective' if test plants inoculated with it developed as abundantly as those inoculated with the control effective strain. A strain was classified as 'ineffective' if the test plants showed as poor develop-

ment as the uninoculated control plants; otherwise a strain was classified as 'intermediate'.

The bacterial strains were treated with bacteriophage by suspending a loopful of cells, taken from a fresh culture on agar slope, in 1 ml. of liquid phage culture, shaking the suspension for a few minutes to homogenize it, and spreading four drops of the suspension on the surface of an agar plate. The plates were incubated at 25° for 12 days. At first there was no visible bacterial growth, because all phage-susceptible organisms were lysed. On the eighth to tenth day single colonies of resistant cells appeared, and by the twenty-first day their growth was sufficient for isolation. After isolation, the growth of the strains was apparently as vigorous as that of the parent strains, i.e. their colonies would be well developed in about 5 days.

RESULTS

The appearance of new features

Fifteen to twenty plates were used with each of the four bacterial strains for the isolation of bacteria that had survived the phage treatment. All isolations were made from single colonies. The numbers of colonies from which isolations were made were 50, 57, 55 and 55 from strains A, B, C and D respectively. Eight strains isolated from phage-treated strain B appeared to be unable to induce nodule formation. Such 'non-infective' mutants have been isolated previously by means of phage treatment from normal nodule-producing strains, and described by Krasil'nikov (1941). In the present work these non-infective mutants were not studied in any detail, and only those that retained the ability to form nodules are dealt with below. The fact that non-infective mutants were obtained only from one of the four tested strains suggests that the tendency to form such mutants varies from one strain to another.

Some of the phage-resistant colonies did not differ in appearance from those of the parent strains, others differed in various respects. Some were typically rough, others smooth or intermediate; some were opaque and others transparent; some were pure white whereas others showed yellow or pink pigmentation. The colonies were composed of Gram-negative rods that did not differ in appearance from those of the parent strains, except for the presence of Gram-positive granules in the rods of most colonies which showed yellow or pink pigmentation.

Table 1 gives the numbers of morphologically new and parent-type colonies obtained from the four tested strains. The figures in brackets show the numbers

Table 1. *The numbers of morphologically changed and unchanged colonies*

(The numbers in brackets are computed by distributing the right-hand marginal totals in proportion to the bottom-line totals. $\chi^2 = 27{\cdot}5$; D.F. $= 3$; $P < 0{\cdot}001$.)

Parent strain	Unchanged	Changed	Total
A	4 (13)	46 (37)	50
B	15 (13)	34 (36)	49
C	9 (15)	46 (40)	55
D	28 (15)	27 (40)	55
	56	153	209

of new and parent type colonies expected if all four strains produced the same percentage of new types. These were calculated by dividing each total shown in the right column in proportion to those in the bottom line. The actual numbers differ from those expected with a χ^2 of 27·5 with 3 degrees of freedom ($P < 0{\cdot}001$), showing that the four strains differ very significantly in their tendency to produce colony mutants.

All the strains isolated from the forty-nine colonies from strain B were tested serologically with an antiserum to the parent strain. Four of them failed to agglutinate.

The results of testing the derivative strains for effectiveness in nitrogen fixation (Table 2) are based on four replicate tubes and show that the four parent strains gave widely different proportions of effective and ineffective mutants. A large proportion of derivatives obtained from the effective

Table 2. *Numbers of derivative strains classified according to their effectiveness in nitrogen fixation*

(Strains classified as uniform produced the same response (effective or ineffective) on all test plants used. Those classified as non-uniform gave different results in the four replicate tubes. The numbers of effective, intermediate and ineffective responses in the individual tubes in this group were respectively 18, 26, 44 for strain A; 3, 5, 20 for strain B; and 1, 4, 15 for strain C.)

	Uniform			
Parent strain	Effective	Ineffective	Non-uniform	Total numbers
A (effective)	17	11	22	50
B (ineffective)	1	41	7	49
C (ineffective)	0	50	5	55
D (ineffective)	0	55	0	55

strain A differed from the parent strain; only small proportions of derivatives obtained from the ineffective strains B and C differed from them, and all the derivatives obtained from the ineffective strain D remained unchanged. It is obvious, therefore, that the tendency to produce mutation in nitrogen fixing effectiveness varies very considerably from one strain to another. Some of the derivative strains behaved uniformly, i.e. gave the same results on all test plants whereas some others did not, i.e. gave different results on different test plants. Experiments designed to examine this non-uniformity are described below.

When the derived strains were tested with the bacteriophage used for their isolation it was found that some were lysed (Table 3). About the same propor-

Table 3. *The numbers of phage-resistant and susceptible derivatives*

(The numbers in brackets computed as in Table 1. $\chi^2 = 2{\cdot}53$; D.F. $= 3$; $P = 0{\cdot}5$.)

	Derivatives		
Parent strain	Resistant	Susceptible	Totals
A	41 (38)	9 (12)	50
B	36 (37)	13 (12)	49
C	38 (41·5)	17 (13·5)	55
D	43 (41.5)	12 (13.5)	55
	158	51	209

tions of derivates obtained from the four parent strains were phage-susceptible. The small value of χ^2 shows that deviations of actual numbers from those computed on the assumption that all the strains producing resistant and susceptible derivatives in the same proportions are not significant.

The presence of phage-susceptible strains among the derivatives isolated by means of phage treatment could be explained as a result of local development of protective substances similar to those that were occasionally demonstrated

Table 4. *The total numbers of derivatives with morphologically changed and unchanged colonies among phage-resistant and susceptible derivatives obtained from all four parent strains*

(The numbers in brackets computed as in Table 1. $\chi^2=2{\cdot}7$; D.F. $=1$; $P=0{\cdot}1$.)

	Morphology of colonies		
	Unchanged	Changed	Totals
Resistant	47 (42·5)	111 (115·5)	158
Susceptible	9 (13·5)	42 (37·5)	51
	56	153	209

Table 5. *The total numbers of derivatives with changed and unchanged effectiveness in nitrogen fixation among phage-resistant and susceptible derivatives obtained from strains A, B and C*

(The numbers in brackets computed as in Table 1. $\chi^2=1{\cdot}06$; D.F. $=1$; $P=0{\cdot}3$.)

	Nitrogen fixation		
	Unchanged	Changed	Totals
Resistant	79 (81·5)	36 (33·5)	115
Susceptible	30 (27·5)	9 (11·5)	39
	109	45	154

in liquid cultures containing phage-susceptible secondary growths (Kleczkowska, 1945*b*). The presence of such protective substances would enable bacteria identical with those of the parent strains to survive and to form colonies. An alternative explanation is that the colonies were composed of bacteria immune to the phage, but the immunity was lost in the course of further growth after the isolations. The second explanation seems to be more probable for two reasons. First, the phage-susceptible derivatives were not identical with the parent strains, for they included forms that differed from the parent strains in colonial morphology and effectiveness of nitrogen fixation. Moreover, Tables 4 and 5 show that approximately the same proportions of derivatives changed and unchanged in colonial morphology and effectiveness in nitrogen fixation, were phage-resistant and susceptible. Secondly, although some of the derivative strains were phage-susceptible, they differed from the parent forms in that their complete lysis took longer and the secondary growth appeared much sooner.

Table 6 showed that mutants in colonial morphology were equally distributed among strains that did and did not mutate in effectiveness of nitrogen fixation. The two mutations, therefore, occurred independently of each other.

Table 6. *The total numbers of derivatives with changed and unchanged effectiveness in nitrogen fixation among those with changed and unchanged morphology of colonies, obtained from strains A, B and C*

(The numbers in brackets computed as in Table 1. $\chi^2=0{\cdot}86$; D.F. $=1$; $P=0{\cdot}35$.)

Morphology of colonies	Nitrogen fixation: Unchanged	Nitrogen fixation: Changed	Totals
Unchanged	21 (19)	6 (8)	27
Changed	88 (90)	39 (37)	127
	109	45	154

When the strains A, B, C and D were plated with bacteriophage to isolate phage-resistant mutants, they were also plated without bacteriophage. All the colonies (500-1000 in number from each strain) were alike in appearance, being greyish white, opaque and of smooth type. Isolations were made from a number of colonies equal to the number of colonies obtained at the same time by phage treatment, and the isolates were tested for effectiveness in nitrogen fixation and for susceptibility to the bacteriophages. All the isolates were identical with the strains from which they were obtained. It can be concluded, therefore, that the strains are normally stable in culture as regards the features under examination, and that it was the phage treatment that revealed the presence of the mutant forms.

Stability of new features

The new morphological features of colonies of derivative strains obtained by phage treatment remained unaltered during up to 3 years' propagation on the agar medium with subculture at 5-6 month intervals. On the other hand, after a single passage through the host plant, most of the strains acquired the colonial appearance of the parent strains from which they have been derived, although in some cases the new characters such as roughness and pink colour were retained after a passage through the nodule.

The newly acquired level of effectiveness in nitrogen fixation was usually retained unaltered during cultivation on media as well as during passages through host plants, by those strains which behaved uniformly when tested the first time, i.e. which were either effective or ineffective on all test plants used. Table 2 shows, however, that the proportion of these uniform derivative strains was rather small. Others behaved non-uniformly, i.e. a proportion of plants on which they were tested responded effectively, some intermediately and the remainder ineffectively. There were, for example, no derivatives from strain C that differed from it in effectiveness and which behaved uniformly.

Strains behaving non-uniformly can be conveniently divided into two groups according to whether uniformly behaving cultures could be isolated from them. All the non-uniform strains from strains A and B belonged to the first group. The cultures isolated from plants that responded ineffectively were all uniformly ineffective. Those isolated from plants that responded effectively (or intermediately) were either all uniformly effective (or intermediate), or some, usually isolated from nodules deliberately chosen for their large size, were

uniformly effective (or intermediate) and those isolated from nodules chosen for their small size, uniformly ineffective. This resembles segregation of mixtures into separate components. Isolations from single nodules usually give pure cultures of single strains when plants are inoculated with mixtures of different strains (Nicol & Thornton, 1941; Hughes & Vincent, 1942).

The five non-uniform strains derived from strain C (Table 2) all belonged to the second group. The cultures obtained from single nodules of the plants on which they were tested, irrespective of the character of their response, were

Table 7. *Total numbers of intermediate and ineffective responses obtained with cultures isolated from each plant inoculated with a different strain derived from strain C*

(The numbers in brackets computed as in Table 1. $\chi^2=8{\cdot}9$; D.F. $=4$; $P=0{\cdot}065$.)

Plant	Number of nodules plated	Intermediate	Ineffective	Totals
1	6	14 (9·6)	46 (50·4)	60
2	6	15 (9·6)	45 (50·4)	60
3	12	13 (19·2)	107 (100·8)	120
4	12	18 (19·2)	102 (100·8)	120
5	9	12 (14·4)	78 (75·6)	90
	45	72	378	450

again non-uniform in their behaviour. Table 7 illustrates the results of experiments leading to this conclusion. One plant showing an effective or intermediate response was chosen from each set of plants infected with each of the five derived strains that behaved non-uniformly. Platings were made from a number (6–12) of nodules from each plant and from each plating ten single colonies were tested on the plant for effectiveness. Columns 3 and 4 give the total numbers of intermediate and ineffective responses (there were no effective responses) obtained with all the colonies derived from nodules of each of the five plants. The cultures from different plants tended to give different ratios of intermediate to ineffective results, but the value of χ^2 shows that this difference may have arisen by chance.

The results obtained individually with the forty-five sets of ten tests, in each of which a culture isolated from each single nodule was tested, are given in Table 8. The total numbers of sets that contained different numbers of plants which gave intermediate responses are shown. Out of the total of forty-five sets distributed amongst the five plants, thirteen gave ineffective responses in all ten tests and the remainder gave different kinds of responses.

Table 7 shows that 16 % of the total of 450 tests gave intermediate responses. If, therefore, all the tested cultures had been identical, the probabilities of a test giving intermediate or ineffective response would be 0·16 and 0·84, respectively. Thus the probabilities that ten replicate tests will contain 0, 1, 2, 3, etc., plants responding intermediately would equal the successive terms of the expanded binomial $(0{\cdot}84+0{\cdot}16)^{10}$. The probabilities are given in Table 8, and the expected numbers of sets of ten plants, out of the total of

Table 8. *Total numbers of sets of ten plants containing different numbers of plants that gave intermediate responses*

($\chi^2 = 8{\cdot}94$; D.F. $= 2$; $P = 0{\cdot}012$.)

Number of intermediate responses	Probability	Number of sets: Expected	Number of sets: Observed
0	0·175	8	13
1	0·333	15	13
2	0·286	13	6
3	0·145	6·5	9
4	0·048	2	1
5	0·011	0·5	2
6	0·0017	0	1
7	0·00018	0	0
8	0·000013	0	0
9	0·00000055	0	0
10	0·000000011	0	0
		9	13
		45	45

forty-five, that would contain different numbers of plants responding intermediately, are obtained from these probabilities. The value of χ^2 for the deviations of actual from the expected numbers shows that the deviations are significant, and hence the chances of giving intermediate or ineffective response in a single test by different cultures were not identical.

Table 9. *Total numbers of intermediate and ineffective responses obtained with cultures isolated from large and small nodules*

(The numbers in brackets computed as in Table 1. $\chi^2 = 0{\cdot}149$; D.F. $= 1$; $P = 0{\cdot}7$.)

	Intermediate	Ineffective	Totals
Large	37 (38·5)	203 (201·5)	240
Small	35 (33·5)	175 (176·5)	210
	72	378	450

Table 9 shows that cultures isolated from nodules marked for their relatively large size did not tend to give results different from those given by cultures isolated from nodules marked for their relatively small size. On another occasion nodules developed during the winter with the same strain C attained a large size while still giving an ineffective response. Sections of these showed that their bacterial tissue was almost wholly disintegrated. (Unpublished observation of Dr P. S. Nutman.)

The non-uniform derivatives retained their diverse behaviour not only on plant passage but also after repeated platings. This is shown by experimental results given in Table 10. Two plants (1 and 2, Table 7), which had served as test plants in the experiment shown in Tables 7–10 to test two of the derived strains, were used as starting material. Ten nodules from each plant were plated and a loopful from each of ten colonies was tested for effectiveness, thus giving 100 tests from the nodules of each plant. These 100 tests are referred to as generation I.

Next, one colony isolate, derived from each of the nodules was plated again, and ten of the resulting colonies tested. This gave a second set of 100 tests referred to as generation II. This procedure was repeated once more, a third 'generation' of cultures being obtained (generation III), and these were similarly tested for effectiveness. Thus three successive platings were made from bacteria ultimately derived from each nodule. The experiment thus examines: (1) the

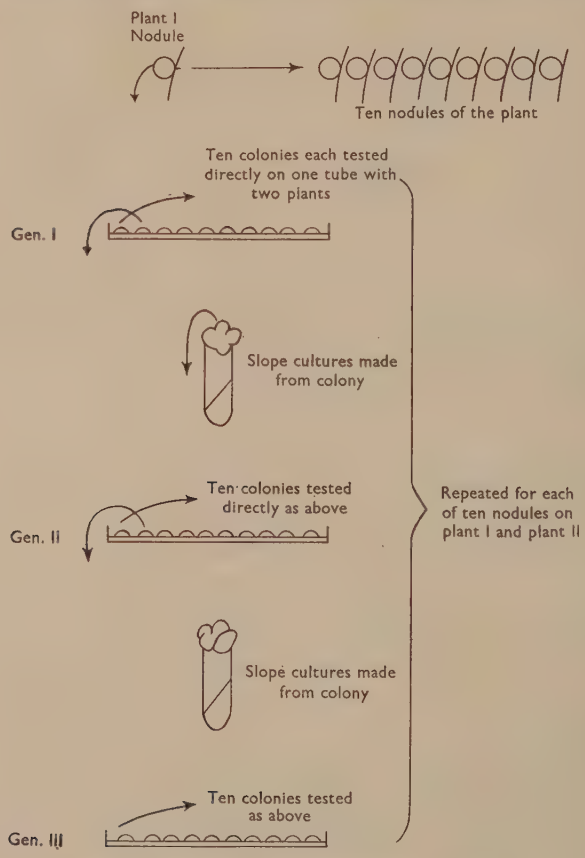


Fig. 1. Plan of the experiment given in Table 10

possibility of altering the proportion of parent and mutant types of bacteria by replating, and (2) the possible differences between the contents of individual nodules as regards the tendency to produce mutants.

The plan of this experiment is set out in the diagram (Fig. 1). Table 10, which gives the results, shows that there was considerable variation between derivatives from different nodules, but the variation between 'generations' was remarkably small. Statistical analysis (see Appendix) showed, however, that because of an interaction between nodules and 'generations' no direct conclu-

Table 10. *The results of testing isolates from single colonies obtained from non-uniform derivatives from strain C*

(The numbers given are of 'intermediate' responses obtained in sets of ten tests with each isolate from a single nodule, the remainder of the set of ten responses being 'ineffective'. A proportion of responses were actually 'effective'. These are added to 'intermediate' responses and the totals are referred to as 'intermediate'.

	Nodules										
'Generation'	1	2	3	4	5	6	7	8	9	10	Totals
					Plant I						
I	3	0	3	5	1	4	3	1	3	1	24
II	2	1	4	2	0	0	5	3	4	4	25
III	4	3	5	0	3	1	0	3	2	3	24
Totals	9	4	12	7	4	5	8	7	9	8	73
					Plant II						
I	7	4	6	1	1	2	3	1	3	2	30
II	4	1	5	5	2	2	4	5	4	1	33
III	1	1	5	4	0	1	9	5	6	7	39
Totals	12	6	16	10	3	5	16	11	13	10	102

sion about the possibility of obtaining uniform behaviour can be drawn from the fact that generation totals varied only slightly. This could be the result of cancelling-out of opposite trends shown by derivatives from different nodules. It is apparent that cultures behaving uniformly could not be obtained by the replating.

In generation III, from plant II, some such possibility was suggested by two sets of ten colony isolates, which, on test, gave 0 and 9 effective responses. The two parent strains from generation III that gave these results were replated, and from each strain sixty colonies were tested. The numbers of effective, ineffective and intermediate responses with the first were 13, 12 and 35, and with the second 15, 17 and 28. The value of the χ^2 for deviations of these numbers from 14, 14·5 and 31·5, which would be expected if the cultures were identical, is 1·78, which for the 2 degrees of freedom, corresponds to the probability of about 0·4. Therefore the behaviour of the two strains was almost identical and none was uniform.

DISCUSSION

The results described above show that phage-resistant mutants tended to be more unstable than the parent strains with regard to various other properties. The newly acquired features remained stable with some mutant strains, but underwent rapid changes with others. An example of this is the rapid loss of immunity to bacteriophage in a proportion of mutant strains. There is also evidence that the strains that became sensitive again acquired immunity more readily than did the untreated strains, since secondary growth in liquid media developed much more rapidly than with the original strains.

The fact that after a single plant passage almost all mutant strains lost their newly acquired colony characters, although retaining them during several years' propagation on laboratory media, suggests a strong selection in the

nodule tissue against colony mutant types. The mutant types differing from the parent strain in effectiveness in nitrogen fixation showed marked differences in stability. A number showed consistent behaviour amongst replicate isolates and retained their new character unaltered on laboratory media and after plant passage. Others did not show this uniform behaviour, and were of two types. Those obtained by phage treatment of strains A and B gave mixed responses at first, but after plant passage and re-isolation from single nodules they could be separated into derived strains that were consistent in giving effective or ineffective responses. This suggests that the original phage-resistant colonies contained mixed populations from which stable mutant types were isolated by plant passage and replating.

The second type of non-uniform derivatives, obtained by phage treatment of strain C, could not be purified either by re-isolation from nodules or by replating, but retained an unimpaired tendency to produce mutants giving effective or intermediate responses. After replating these derived strains, every colony tested was found to contain mutant forms in proportions that remained high after successive platings. Since an examination of the composition of a nodule involved plating the data do not enable us to tell how far mutant forms, which certainly appear in the colonies, also develop in the nodule tissue. The evidence does show, however, that these derived strains are highly unstable and that this instability is retained both on replating and on plant passage.

For reasons given previously (Kleczkowska, 1945*b*), it seems unlikely that phage can directly cause a failure of legume crops by destroying the nodule bacteria. It is possible, however, that phages may increase the proportion of ineffective strains in the soil under certain conditions and this possibility is enhanced by the observation that ineffective phage-resistant mutants are much more readily developed from effective parent strains than vice versa.

The author wishes to thank Dr H. G. Thornton, F.R.S., for his interest in this work and for aid in preparation of the manuscript and Dr A. Kleczkowski for statistical examination of the results.

APPENDIX

The proportions of 'intermediate' responses, shown in Table 10, were transformed into the angular degrees and subjected to analysis of variance, the results of which are given in Table 11. As ten tests were made with each 'generation' of each strain derived from a single nodule, the theoretical value of the variance is $820{\cdot}7/10 = 82{\cdot}07$. The 'residual' variance (obtained in the analysis of variance by subtracting from the total sum of squares of deviations from the mean those due to overall differences between derivatives from different nodules and between different 'generations'), is, in the case of both plants, significantly greater than the theoretical variance ($P < 0{\cdot}001$). This can be interpreted as an evidence of interaction between the origin from different nodules, on the one hand, and different 'generations', on the other. Thus, the tendencies to produce an 'intermediate' or 'ineffective' response probably

Table 11. *The analyses of variances of the results given in Table* 10

(The proportions of 'intermediate' responses were transformed into angular degrees.)

		Plant I		Plant II	
	D.F.	Sums of squares	Mean square (variance)	Sums of squares	Mean square (variance)
Between nodules	9	1482·95	164·77	2919·02	324·34
Between 'generations'	2	5·21	2·60	88·20	44·10
Residual	18	4586·93	254·82	4072·07	226·23
Total	29	6075·09		7079·29	
Theoretical variance	∞		82·07		82·07

changed from one 'generation' to another, differently with derivatives of different nodules, and, consequently, no direct conclusions can be drawn from 'generation' totals (of untransformed data or of the transformed proportions).

Variation between nodules is highly significant with derivatives from both plants ($P = 0{\cdot}05$ for plant I and $P < 0{\cdot}001$ for plant II).

REFERENCES

Allen, O. N. & Baldwin, I. L. (1931). The effectiveness of Rhizobia as influenced by passage through the host plant. *Bull. Wis. agric. Exp. Sta.* no. 106.

Almon, L. & Baldwin, I. L. (1933). The stability of cultures of *Rhizobium*. *J. Bact.* **26**, 229.

Burnet, F. M. (1929). Smooth-rough variation in bacteria in its relation to bacteriophage. *J. Path. Bact.* **32**, 15.

Frank, B. (1899). Die bisher erzielten Ergebnisse der Nitraginimpfung. *Landw. Vers.Sta.* **51**, 441.

Hughes, D. Q. & Vincent, J. M. (1942). Serological studies of the root-nodule bacteria. III. Test of neighbouring strains of the same species. *Proc. Linn. Soc. N.S.W.* **67**, 1942.

Hutchinson, C. M. (1924). Report of Imperial Agricultural Bacteriologist. III. Soil biology. *Sci. Rep. agric. Res. Inst. Pusa*, **32**.

Kleczkowska, J. (1945*a*). The production of plaques by *Rhizobium* bacteriophage in poured plates and its value as a counting method. *J. Bact.* **50**, 71.

Kleczkowska, J. (1945*b*). A quantitative study of the interaction of bacteriophage with *Rhizobium* using the technique of poured plates. *J. Bact.* **50**, 81.

Krasil'nikov, N. A. (1941).Variability of nodule bacteria. I. The origin of strains under the influence of bacteriophage. *Mikrobiologia*, **10** (4), 396.

Luria, S. E. & Delbrück, M. (1943). Mutations of bacteria from virus sensitivity to virus resistance. *Genetics*, **28**, 491.

Newcombe, H. B. (1949). Origin of bacterial variants. *Nature, Lond.*, **164**, 150.

Nicol, H. & Thornton, H. G. (1941). Competition between related strains of nodule bacteria and its influence on infection of the legume host. *Proc. Roy. Soc.* B, **130**, 32.

Nobbe, F. & Hiltner, L. (1893). Wodurch werden die Knöllchenbesitzeden Leguminosen befähigt den freien atmospharishen Stickstoff für sich zu verwerten? *Landw. Vers.Sta.* **42**, 459.

Nutman, P. S. (1946). Variation within strains of clover nodule bacteria in the size of nodule produced and the 'effectivity' of the symbiosis. *J. Bact.* **57**, 41.

Simon, J. (1908). Die Widerstandsfähigheit des Wurzelbakterien der Leguminosen und ihre Bedeutung für die Bodenimpfung. *Angew. Bot.* **5**, 132.

Snieszko, S. (1929). Beitrage zur Kenntnis der Zelluloze zersetzenden Bakterien. *Zbl. Bakt.* Abt. II, **78**, 375.

Stapp, C. (1929). Zur Frage der Planmässigen Erteilung hochwirksamer Leguminosen-Knöllchenbakterienkulturen. *Angew. Bot.* **11**, 197.

Virtanen, A. I. (1945). Symbiotic nitrogen fixation. *Nature, Lond.*, **155**, 747.

(*Received* 22 *October* 1949)

An Investigation of the Biological Properties of Organisms of the Pleuropneumonia Group, with Suggestions Regarding the Identification of Strains

By D. G. ff. EDWARD

The Wellcome Veterinary Research Station, Frant, Sussex

SUMMARY: Seventeen representative strains of pleuropneumonia-like organisms were investigated to determine whether they could be identified by cultural methods.

The L1 organism was readily identified by its morphology and by its cultural appearances on solid and in fluid and semi-solid media. Differences, depending on whether growth occurred anaerobically, whether growth was smooth or granular and whether a precipitate was formed in horse serum media, served to distinguish most of the other strains.

The requirements of serum and yeast extract for promoting growth and the effect of pH were studied with each strain.

Several strains fermented carbohydrates. Young cultures of some strains reduced methylene blue. Haemolysis around colonies in horse blood agar was noted with some strains; young broth cultures of these strains discoloured suspensions of horse erythrocytes. The factor discolouring erythrocytes was found in filtrates of old cultures of *Asterococcus bovis*.

Two strains were pathogenic for mice; when recovered after passage they showed no alteration in their nutritional requirements.

The organisms were resistant to penicillin; one strain grew in a medium containing 3000 units/ml.

In 1898 the filtrable agent causing contagious bovine pleuropneumonia was cultivated in a cell-free medium by Nocard & Roux, and in 1910 the peculiar morphology of this organism was described by Bordet and by Borrel, Dujardin-Beaumetz, Jeantet & Jouan. In 1923 agalactia in sheep was shown to be due to an organism of similar morphology. Within recent years a number of other similar organisms have been isolated. Some were obvious saprophytes, others were the cause of disease in animals; the pathogenicity of many, however, is still doubtful.

The group of pleuropneumonia-like organisms are characterized by their peculiar morphology and colonial appearances. Although capable of cultivation in artificial media, the smallest elements pass filters which will retain ordinary bacteria. These properties have distinguished them from bacteria. The group also includes similar organisms which have been isolated from cultures of bacteria. Although originally regarded by Klieneberger (1936, 1940) as symbionts, recent investigations suggest that they are variants of the bacteria. A classification and nomenclature for the group suggested by Sabin (1941*a*, *b*) has not been generally accepted, and would seem to be premature until the relationship of the group to the bacteria has been elucidated. There appear to be a number of species, identified by Sabin according to habitat and pathogenicity, each distinct antigenically. Until a nomenclature has been agreed the

species and the existing strains must be referred to by the symbols given them when they were isolated.

Although the organisms causing bovine pleuropneumonia and agalactia are important in veterinary medicine, the other members of the group have aroused interest more as bacteriological curiosities. The recent isolation, however, of organisms of this group from human (Dienes, 1940; Dienes, Ropes, Smith, Madoff & Bauer, 1948) and bovine (Edward, Hancock & Hignett, 1947) genital tracts suggests that the group may acquire a greater importance. It has yet to be determined whether the organisms inhabiting the genital tract are pathogenic and the problem is made more difficult by a lack of knowledge regarding the general cultural and metabolic properties of the pleuropneumonia-like organisms. More than one species of pleuropneumonia-like organism may reside in the same situation. Sabin (1939) found several species with distinct serological and pathogenic properties inhabiting the respiratory tract of mice, and two different species were isolated from the genital tract of cattle (Edward, 1950). In the examination of material it is therefore necessary not only to determine that a pleuropneumonia-like organism is present but to identify the species.

Few attempts have been made to examine and compare a number of strains from different sources by the same methods. The most detailed observations were made by Klieneberger (1938, 1940), but they were largely confined to morphology, colonial appearances and antigenic constitution. Warren (1942) also compared the biochemical and metabolic activities of a number of strains. The present investigation was an attempt to distinguish between strains, representative of different species within the pleuropneumonia group, by examining those cultural properties which are commonly used in the identification of bacteria.

TECHNICAL METHODS

A total of seventeen strains were available for investigation; relevant details are given in Table 1. They were collected from laboratories in this country and in the U.S.A., and represent as many members as possible of the pleuropneumonia group. Unfortunately, cultures of several reported species are no longer in existence.

Strains were maintained by weekly subculture on plates of a medium made from ox-heart infusion broth, containing 1 % peptone, to which was added 10 % of a freshly prepared yeast extract and 20 % of horse serum, the reaction being finally adjusted to pH 8·0. Contamination by other bacteria was avoided by the use of thallium acetate and penicillin (Edward, 1947*a*). The plates were incubated aerobically at 37° for 3 days in closed tins containing exposed pieces of moist cotton-wool, thus ensuring a moist atmosphere throughout the period of incubation. Plates were inoculated by cutting out a small piece of agar bearing growth, which was then inverted over the plate to be inoculated and smeared across it.

For various investigations modifications, which will be described later, were made to this medium. A fluid medium (without agar) and a semi-solid medium (containing 0·3 % agar), were also used. Two strains, L3 and L5, could not be

Table 1. *Details of strains investigated*

Strain	Details	References
Nos. 3278, 4159, 4732	Strains of the organism causing contagious bovine pleuropneumonia (*Asterococcus bovis*)	From The National Collection of Type Cultures
Sewage A and B	Saprophytic strains, serologically different, isolated from sewage*	Laidlaw & Elford (1936); received from Dr W. J. Elford
L1 (Rat 30)	Isolated from a culture of *Streptobacillus moniliformis*	Klieneberger (1936, 1940); received from Dr E. Klieneberger-Nobel
L3 (Ash)	Isolated from bronchiectatic lesions in a rat. Produced local abscesses in mice	Klieneberger & Steabben (1940); received from Dr E. Klieneberger-Nobel
L4 (Preston)	Isolated from rats; produced abscesses and purulent arthritis. Has the pathogenic properties of L4, although its antigenic identity with the original L4 strain has not been proved	Klieneberger (1938, 1939); Preston (1942); received from Dr L. Dienes
L5	Isolated from mice; caused 'rolling disease'	Findlay *et al.* (1938); received from Dr E. Klieneberger-Nobel
M46	Isolated from mice suffering from infectious catarrh	Edward (1947*b*)
C140	Isolated from mice; pathogenicity not known	Received from Dr L. Dienes
H17	From the human genital tract; isolated from a cervical swab	Isolated in this laboratory
92, 93	Isolated from the human genital tract	Received from Mr S. R. M. Bushby
B2	A P strain isolated from the bovine genital tract	Edward (1950)
B30	A P strain from the bovine genital tract which had received only a few subcultures	Edward (1950)
B15	An S strain isolated from the bovine genital tract	Edward (1950)

* The third serological type (sewage C), isolated from sewage, was unfortunately no longer available. The saprophytic strains isolated by Seiffert (1937) were closely related to sewage A (Klieneberger, 1940).

cultivated on the medium in routine use. They were inhibited by a 10 % concentration of yeast extract and for their cultivation media containing 0·5–1 % yeast extract were employed.

RESULTS

Colonial appearances of surface cultures

The surface colonies of organisms of the pleuropneumonia group are so characteristic that their examination with a dissecting microscope (magnification × 10) provides the most convenient and reliable method for identifying an unknown organism as a member of the group, studies of its morphology in stained preparations and in unstained preparations examined by the dark-ground microscope affording confirmation.

The typical colony was hemispherical, sometimes slightly flattened, often with a central papilla or depression. When examined magnified × 10 the most

characteristic feature was a central spot, light brown in colour, produced by growth into the medium. The colony was translucent with a circular outline and the surface was either faintly marked with irregular lines or appeared finely pitted.

Colonies of the L1 organism, although having the same general characters, were quite distinctive. They were more opaque and were of a light brown colour, the central spots being a darker brown; their surface was coarsely marked with black lines. Therefore, although having the characteristic central spot, in some respects they resembled colonies of ordinary bacteria. L1 also gave a bacterial type of growth in fluid and semi-solid media. This strain was the only pleuropneumonia-like organism, isolated from a bacterial culture, which was available for examination; it would be interesting to determine whether other strains derived from bacterial cultures have cultural characters which are in part those of a pleuropneumonia-like organism and in part those of the parent bacterium, thus distinguishing them from the other members of the group.

Although colonies of all the other strains had the general appearance described, detailed examination revealed minor differences. For instance, strains differed in the maximum size attained by isolated colonies, and the relative size of the central spot, compared to the size of the whole colony, varied. There were also slight differences in the appearances of the central spots and in the manner in which they were demarcated from the rest of the colony. These differences sometimes sufficed to identify colonies of a particular strain. On the other hand, there were differences between the colonial appearances of the three strains of the one species, *Asterococcus bovis*. It is therefore unlikely that colonial appearances, which possibly also alter according to the conditions of cultivation, could be used to identify an unknown strain.

The production of certain characteristic effects by P strains, isolated from the bovine genital tract, after 5 days' growth on media enriched with horse serum has been described elsewhere (Edward, 1950). When viewed by the dissecting microscope numerous small black dots could be seen in the upper layer of the medium beneath and around the colonies, and there was often a crinkled film on the surface of the medium. Similar changes were produced by the strain L3 but not by any of the others. They are therefore important in the identification of the P and L3 organisms.

Cultural appearances in fluid and semi-solid media

Information of greater value for identifying members of the pleuropneumonia-group was obtained by examining cultures in a semi-solid medium (Beveridge, 1943). Some organisms grew as well anaerobically as aerobically, so that growth was equal throughout a tube of the medium; others only grew near the top where conditions were aerobic or micro-aerophilic. There were also differences depending on whether growth was smooth or granular.

The three strains of *A. bovis* only grew within 2 cm. from the top of the medium. Colonies were fluffy and had poorly defined margins. When the inoculum was heavy, growth produced a generalized opacity in the upper part

of the medium. Growth of the saprophytic strains (Sewage A and B, and B15) was similar and also confined to the upper 2 cm. of the medium. All P strains grew equally well throughout the medium, colonies being fluffy and diffuse with ill-defined margins. When the inoculum was heavy a generalized fluffy opacity developed in the medium, the opacity being greater than with most of the other organisms. The growth of the L4 organism was similar to that of the P strains, except that it was less opaque. When grown in a fluid medium, all the above-mentioned strains produced a uniform opalescence, with or without a small deposit. The growth of these strains in both fluid and semi-solid media was thus predominantly smooth.

The other strains gave granular growths. Even when the inoculum was heavy the human genital strains (H17, 92 and 93) grew in semi-solid media as small white circumscribed masses, which were dense and opaque, sometimes surrounded by a narrow fluffy halo. Colonies developed throughout the medium. Growth of the mouse strains, M46 and C140, was similar. The ability of M46, however, to grow anaerobically had only developed during artificial cultivation, as, when examined soon after isolation, it grew only near the surface (Edward, 1947*b*). Strains L3 and L5 produced a similar type of growth which was confined to the upper part of the medium. In fluid media all these strains grew as a deposit of small dense white masses, the supernatant fluid remaining clear.

Cultures of the L1 organism were quite distinctive. In the semi-solid medium colonies were found only near the surface and were relatively large, spherical and well-circumscribed; they were densely opaque and usually pigmented brown or black. In a fluid medium growth occurred as large spherical masses, densely opaque and often pigmented, which formed a deposit.

Morphology

The centrifuged deposits of horse-serum broth cultures were examined by dark-ground microscopy. Stained preparations of surface colonies were also examined. All strains exhibited the peculiar pleomorphic morphology characteristic of the pleuropneumonia group. The L1 organism differed from the others. Under dark-ground illumination the majority of its elements were thicker and more refractile. Cultures of the P strains and of the L3 organism were also distinctive, because they contained peculiar spherical masses which could be stained by Giemsa and were highly refractile under dark-ground illumination (Edward, 1950). There were no significant differences between the appearances of the other strains to assist identification.

Growth requirements

Only the three saprophytic strains, B15 and Sewage A and B, grew at room temperature. These three strains also grew well on infusion agar base without the addition of yeast extract or serum. Enrichment with an animal protein, such as serum, was needed for the multiplication of all other strains; 10 % of horse serum was the least amount which regularly gave maximal growth of all

strains. Ox serum, rabbit serum and human ascitic fluid were compared with horse serum for their ability to promote growth. Most of the strains had been maintained for long periods on horse-serum media and grew best with horse serum, although some strains gave equally good growth with rabbit serum or ascitic fluid. Ox serum gave poor results with most strains. One strain, received from Dr L. Dienes, who had maintained it on ascitic fluid media, grew best with ascitic fluid and only poorly with horse serum. The results were interpreted as confirming the opinion of Sabin (1941*a*), who believed that during subculture organisms of the pleuropneumonia group become adapted to the protein of a particular species of animal. Thus, although strains differed in the way the various sera promoted their growth, the differences were probably properties of the strain itself, depending on how it had been maintained, and could not be used to differentiate between members of the group.

Incubation of plate cultures in a McIntosh & Fildes' jar confirmed that anaerobic conditions prevented or impaired the growth of those strains which only grew near the surface of a semi-solid medium. An atmosphere of 10 % carbon dioxide did not improve the growth of any strain; it impaired the growth of the three strains of *A. bovis*, the three saprophytic organisms, the P strains and L1, and completely inhibited L3 and L5.

Effect of yeast extract. The addition of fresh yeast extract has been found to improve media used for cultivating this group of organisms (Edward, 1947*a*). The growths of all strains on horse-serum agar plates, containing 10, 5, 2, 1, 0·5 % and no yeast extract were compared. Yeast extract did not improve the growth of the three saprophytic organisms, L1 and strain no. 3278 of *A. bovis*, but in a minimum concentration, varying between 10 and 1 % effected a marked improvement in the growth of strains L4, M46, C140, B2 and H17.

Two strains, L3 and L5, were peculiar in being inhibited by a high concentration of yeast extract (Table 2). A smaller concentration, 0·5–1 %, enhanced the

Table 2. *The effect of yeast extract on growth; enhancement and inhibition*
(+, maximal growth; ±, moderate growth only; Tr, slight growth only; —, no growth.)

	Yeast extract present (%)					
	0	0·5	1	2	5	10
Organism	Effect on growth					
L3	±	+	+	±	Tr	—
L5	+	+	+	+	±	±
L4*	Tr	±	±	+	+	+

* Included as an example of an organism, whose growth was not inhibited, but only enhanced by yeast extract.

growth of L3. The nature of the inhibiting factor was not ascertained, and it is not known whether it was identical with the factor promoting growth; neither factor was soluble in ether. It is probable that the sensitivity of these two strains developed during maintenance in culture. Both were strains recently received from Dr E. Klieneberger-Nobel and had been maintained by subculture for several years. When a subculture of the same strain of L5 was

examined 2 years ago, its growth was then shown to be improved by addition of 10 % yeast extract to the medium, there being no inhibition (Edward, 1947*a*).

Effect of pH. The effect of pH was studied by adjusting the final reaction of the medium to values between pH 6·8 and 9·2. The following strains were tested: *A. bovis* (3 strains), Sewage A and B, B15, L1, L3, L4, L5, H17, B2 and M46. All except M46 and strain no. 4159 of *A. bovis* gave maximal growth in the range pH 7·6–8·4. M46 was slightly impaired by pH 7·6 and no. 4159 by pH 8·4. The results confirm the necessity for using as a routine a medium adjusted to pH 8·0. However, some of the strains gave maximal growth over a wide range of pH. The strains of *A. bovis* and the L1 organism grew well at pH 6·8; growth of B2, L3 and L5 was not impaired at pH 8·8 and that of Sewage A not even at pH 9·2.

Fermentation of carbohydrates. A. bovis was shown to ferment certain carbohydrates, with the formation of acid only, by Tang, Wei, McWhirter & Edgar (1935). Warren (1942) demonstrated the production of acid from glucose only by L1, L3, L4 and L5. Laidlaw & Elford (1936) could not detect any fermentation of carbohydrates by the organisms they isolated from sewage. Similarly, Edward (1940) failed to demonstrate the production of acid from carbohydrates by an organism isolated from a transmissible pneumonia in mice. Apart from these observations there appears to have been no investigation of the fermentative properties of the pleuropneumonia group.

The organisms were grown on the surface of horse-serum agar plates, to which had been added 1 % of carbohydrate and 0·005 % of phenol red. Cresol red, also giving a change of colour between pH 7 and 8, had no advantage as an indicator. In a few confirmatory tests there appeared to be no advantage in carrying out the tests in fluid or semi-solid media. In all the tests control plates were included, containing phenol red but no added carbohydrate.

Altogether fourteen strains were examined and with eight the production of acid from certain carbohydrates was demonstrated (Table 3). The two sewage organisms fermented glucose, maltose, fructose, dextrin, starch and glycogen. Strain B15, an S strain from the bovine genital tract, gave similar reactions, except that acid was formed from galactose and not from fructose.

The L1 organism fermented glucose, maltose, fructose, galactose, mannose, dextrin, glycogen and salicin. Salicin, which is also fermented by *Streptobacillus moniliformis* (Topley & Wilson, 1946), was not fermented by any other strain. Strains L3 and L5 fermented glucose, maltose, mannose, dextrin, starch and glycogen; it should be noted that mannose was not fermented by the saprophytic strains. One strain of *Asterococcus bovis* (no. 4159) behaved similarly to L3 and L5, except that there was also a weak fermentation of fructose. Another strain of this organism (no. 4732) did not ferment any carbohydrate; it is possible that its fermentative properties had been lost during long maintenance in culture. The third strain (no. 3278) gave weak and variable fermentations. Strains B2, H17, M46 and C140 and L4 did not ferment any carbohydrate. None of the strains fermented lactose, sucrose, mannitol, dulcitol, rhamnose, xylose, trehalose, raffinose, sorbitol, inulin and inositol.

Table 3. *Fermentation of carbohydrates*

(+, fermentation with the production of acid; ±, weak or variable fermentation; —, no fermentation; ., no observation made.)

Strain	Glucose	Fructose	Galactose	Maltose	Mannose	Dextrin	Starch	Glycogen	Salicin	Lactose	Sucrose	Mannitol	Dulcitol
Sewage A	+	+	—	+	—	+	+	+	—	—	—	—	—
Sewage B	+	+	—	+	—	+	+	+	—	—	—	—	—
B15	+	—	+	+	—	+	+	+	—	—	—	—	—
Asterococcus bovis (no. 4732)	—	—	—	—	—	—	—	—	—	—	—	—	—
A. bovis (no. 3278)	+	+	—	±	+	±	—	—	—	—	—	—	—
A. bovis (no. 4159)	+	±	—	+	+	+	+	+	—	—	—	—	—
L1	+	+	+	+	+	+	—	+	+	—	—	—	—
L3	+	—	—	+	+	+	+	+	—	—	—	—	—
L5	+	—	—	+	+	+	+	+	—	—	—	—	—
L4	—	—	—	—	.	—	.	.	—	—	—	—	—
B2	—	—	—	—	.	—	.	.	—	—	—	—	—
H17	—	—	—	—	.	—	.	.	—	—	—	—	—
M46	—	—	—	—	.	—	.	.	—	—	—	—	—
C140	—	.	.		.	.	.	.	.	.	.	.	.

Other metabolic characters

Reduction of methylene blue. Holmes & Pirie (1932) showed that cultures of *A. bovis* reduced methylene blue. Warren (1942) reported experiments which suggested that during repeated subculture of an L4 organism the ability to reduce methylene blue was lost in parallel with a decrease in virulence. Methylene-blue reduction tests were carried out on the strains under investigation by incubating, in $2\frac{3}{4} \times \frac{3}{8}$ in. tubes at 37°, 2 ml. of horse-serum broth cultures, to which had been added 0·4 ml. of a 1/10,000 solution of methylene blue. Cultures were examined each day after inoculation.

Reduction of methylene blue was demonstrated with the following strains: *A. bovis* (three strains), sewage organisms (two strains), B15, B2, L1, L3 and L5. No reduction was detected with L4, H17, C140, M46 (Table 4). Only young cultures reduced methylene blue, the greatest degree of reduction being noted when multiplication of the organism was maximal. This was particularly noticeable with the culture of B2. At the end of 24 hr. there was no apparent growth and the test was negative. At two days the medium was very faintly opalescent and the test was positive. By the third day the opacity of the medium had increased greatly but the test was then no longer positive. A positive methylene-blue test persisted longer in cultures of *A. bovis*, becoming weakly positive or negative at the end of a week. This may be associated with a longer viability of this organism in broth cultures.

Haemolysis. Warren (1942) showed that filtrates of *A. bovis* 'reduced' haemoglobin; filtrates of other strains were inactive, although there was sometimes a green discoloration around colonies of L3 on a sheep-blood medium. Haemolysis produced by colonies of bovine P and S strains and of the sewage organisms was described by Edward (1950). In the present investigation strains were tested for haemolytic activity by two methods. First, haemolysis was noted around colonies on horse-blood agar. When pour plates were made on the organism was grown on the surface of this medium, it was not always possible to determine the result, owing to the discoloration which frequently occurred after incubation for several days. It was preferable to grow the culture on the surface of the ordinary horse-serum agar medium for 2 days and then to pour on top a thin layer of the same medium containing 5 % of a horse red cell suspension. The results were read after incubation for a further 2 days. In the second method the strains were grown in horse-serum broth; each day 2 drops of a 5 % suspension of horse erythrocytes were added to 1 ml. of culture. The mixtures were incubated at 37° for 5 hr., being shaken at about half-hourly intervals and examined for changes in the colour of the erythrocytes.

On blood agar plates well-marked haemolysis was noted with the following strains: *A. bovis* (three strains), sewage organisms (two strains), B15, B2, L3, L4 and L5. Strain C140 produced less intense haemolysis. Although there was slight clearing or discoloration round colonies of strains H17, M46 and L1, there was no definite zone of haemolysis, such as was produced by the other strains.

When broth cultures of certain strains were incubated with horse erythro-

Table 4. *Results of tests of methylene blue reduction and of cultivation in colour of erythrocytes*

(Degrees of growth recorded as: + + +, + +, +, ±, relative degrees of visible growth; —, no growth visible. Methylene blue reduction recorded as: +, complete reduction; ±, incomplete reduction; —, no reduction. Changes in colour of erythrocytes recorded as: +, well-marked change; ±, slight change only; —, no change.)

Strain	1st day: Visible growth	1st day: Methylene blue reduction	1st day: Change in colour of erythrocytes	2nd day: Visible growth	2nd day: Methylene blue reduction	2nd day: Change in colour of erythrocytes	3rd day: Visible growth	3rd day: Methylene blue reduction	3rd day: Change in colour of erythrocytes
Asterococcus bovis	+ +	+	·	·	·	·	+ + +	+	+
Sewage organisms	+ + +	+	+	+ + +	+	+	+ + +	—	—
B15	±	+	+	+ + +	+	+	+ + +	—	—
L1	—	—	—	+ +	+	—	+ + +	+	—
L3	—	—	—	+	±	+	+ +	+	+
L4	—	—	—	+	—	—	+ +	—	±
L5	—	—	—	+	—	—	+ +	+	+
M46	—	—	—	—	—	—	+	—	—
C140	—	·	·	—	—	—	—	—	—
H17	—	—	—	—	—	—	+	—	—
B2	—	—	—	±	+	—	+ + +	—	+

Strain	4th day: Visible growth	4th day: Methylene blue reduction	4th day: Change in colour of erythrocytes	5th day: Visible growth	5th day: Methylene blue reduction	5th day: Change in colour of erythrocytes	6th day: Visible growth	6th day: Methylene blue reduction	6th day: Change in colour of erythrocytes
Asterococcus bovis	·	·	·	+ + +	+	+	+ + +	—	+
Sewage organisms	+ + +	—	—	·	·	·	·	·	·
B15	+ + +	—	—	·	·	·	·	·	·
L1	+ + +	—	—	·	·	·	·	·	·
L3	+ +	—	+	+ +	—	+	+ +	—	+
L4	+ +	—	—	+ +	—	—	·	·	·
L5	+ + +	—	+	+ + +	—	—	+ + +	—	—
M46	+	—	—	+ +	—	—	·	·	·
C140	—	—	—	+	—	—	·	·	·
H17	+ +	—	—	+ +	—	—	·	·	·
B2	+ + +	—	—	·	·	·	·	·	·

cytes, the suspension became a greenish brown colour after a few hours. There was no haemolysis; the cells, in fact, became more resistant and suffered no haemolysis even after 24 hr. incubation, whereas unaltered cells in control mixtures were haemolysed. The colour change was presumably due to formation of methaemoglobin and was not a simple reduction of haemoglobin. Greenish brown discoloration of erythrocytes was produced by *A. bovis* (three strains), sewage organisms (two strains), B15, B2, L3 and L5. Strain L4 caused slight discoloration only. Strains H17, M46, C140 and L1 were inactive. The ability to discolour erythrocytes persisted in cultures of *A. bovis* and L3 for at least 6 days, but only young cultures of the other strains were active (Table 4). Reduction of methylene blue was also a property of young cultures only, but the two activities were caused by different enzymes. A few cultures reduced methylene blue but did not affect erythrocytes or vice versa.

Effect of centrifugation and filtration on the methylene-blue test and on 'haemolysis'. Experiments were carried out to determine whether the substances responsible for reducing methylene blue and affecting haemoglobin were associated with the organisms themselves or were liberated into the medium. Cultures of both sewage organisms after 24 hr. incubation were centrifuged for 60 min. at full speed in a bench angle centrifuge. Although the original cultures reduced methylene blue rapidly and produced the characteristic change of colour with horse erythrocytes, the supernatants were without effect either on the methylene blue or on the erythrocytes. When resuspended in saline the sedimented organisms were strongly positive in both tests (Table 5). Cultures of *A. bovis,* B2, L3 and L5 were similarly examined after 3 days' incubation at which time they reduced methylene blue and affected erythrocytes. The supernatants proved inactive in both tests. Therefore, with these organisms at this stage of growth, the enzymes responsible for reducing methylene blue and affecting haemoglobin were associated with the intact living cell.

Although with continued incubation cultures of the sewage organisms, L3 and B2 lost the ability to reduce methylene blue and to affect haemoglobin, 6-day cultures of *A. bovis* and L3 still discoloured erythrocytes. The factor affecting erythrocytes was not present in the supernatant after centrifuging a 6-day culture of L3. When, however, a similarly aged culture of *A. bovis* was centrifuged, the supernatant, although no longer capable of reducing methylene blue, still discoloured erythrocytes, the reaction being slower than with the original culture. The filtrate, obtained by filtering the supernatant through a gradocol membrane of 300 mμ. A.P.D. and shown to be sterile by failure to get a positive subculture from an inoculum of 1 ml., affected erythrocytes to the same degree as the original supernatant. It thus appears that although reduction of methylene blue is brought about by enzymes present in the intact organism, the substances affecting red cells are liberated from *A. bovis* during autolysis and can be demonstrated in filtrates. In this respect *A. bovis* behaves differently from the other pleuropneumonia-like organisms.

Table 5. *Effect of centrifugation and filtration on the enzymes reducing methylene blue and affecting erythrocytes*

(Methylene blue reduction recorded as: +, rapid reduction; —, no reduction. Change in colour of erythrocytes recorded as: ++, rapid change; +, slower change; ±, slight change only; —, no change.)

		Original culture		Supernatant after centrifugation		Gradocol filtrate of supernatant		Resuspended, centrifuged deposit	
Strain	Age of culture in days	Methylene blue reduction	Change in colour of erythrocytes	Methylene blue reduction	Change in colour of erythrocytes	Methylene blue reduction	Change in colour of erythrocytes	Methylene blue reduction	Change in colour of erythrocytes
Sewage A	1	+	++	—	—	—	—	+	++
B2	3	+	+	—	—	.	.	—	—
L3	3	+	±	—	—	.	.	—	—
L3	6	—	++	—	—	.	.	—	±
L5	3	+	++	—	—	.	.	—	±
Asterococcus bovis (no. 3278)	3	+	++	—	—	.	.	+	+
	6	+	++	—	+	.	+	+	+

Pathogenicity

Only with a few strains was it possible to investigate pathogenicity in laboratory animals. The mouse strains L5, M46 and C140 were no longer pathogenic, but pathogenic effects were obtained with two strains, both originally isolated from rats. Cultures of the L3 organism in a semi-solid medium produced local abscesses when injected subcutaneously into mice. Serial subcutaneous passage was carried out, using pus as inoculum. The abscesses were small and localized to the site of inoculation; they soon discharged through the skin and the condition cleared up. The pus was without effect when inoculated intracerebrally into mice.

Cultures of the L4 organism were inoculated intravenously and intraperitoneally into rats; intra-abdominal abscesses resulted from the intraperitoneal injections and pus from these produced local abscess formation in mice when inoculated by the subcutaneous and intraperitoneal routes. The subcutaneous abscesses were more diffuse and caused greater induration of surrounding tissue than those due to L3. The intracerebral inoculation of mice with pus, or with cultures of the original organism, caused cerebral symptoms in about 3 days, followed by death, abscesses being formed in the brain. In no experiment did strain L4 cause arthritis either in rats or mice. It appeared that the strain, although retaining its ability to cause abscesses, had lost much of its original virulence and no longer tended to localize in joints.

Reinvestigation of strains after passage in animals

No opportunity occurred to investigate strains immediately after their isolation. The L3 and L4 strains were therefore re-examined after recovery from animals, in order to find out whether their properties had been altered by animal passage. The L4 organism was recovered after four intracerebral passes in mice. At its first subculture growth was enhanced by yeast extract to the same degree as the original strain. Yeast, however, was not necessary for maximal growth when the infected brain suspension itself was plated, probably because of nutritional factors supplied by the inoculum.

The L3 organism was recovered from pus after three passes in mice. Both at the original plating of pus and at the first subculture the organism had the same nutritional requirements as the original strain. Growth of both was inhibited by a high concentration of yeast extract and enhanced by a smaller. Both grew well on horse-serum agar, but not on rabbit-serum or human ascitic-fluid agar. A few passages in animals had therefore not altered the peculiar nutritional requirements of this strain.

Resistance to the bacteriostatic action of penicillin

While using penicillin to obtain pure cultures of pleuropneumonia-like organisms from material contaminated with bacteria it became apparent that organisms of the pleuropneumonia group were extremely resistant and could resist concentrations which would inhibit the most resistant bacteria. Penicillin

has also been used to isolate pleuropneumonia-like variants from certain bacteria by Dienes (1949) who demonstrated multiplication of a variant of this type in media containing 10,000 u. penicillin/ml. It was therefore of interest to determine the highest concentration of penicillin in which growth of a typical pleuropneumonia-like organism could occur.

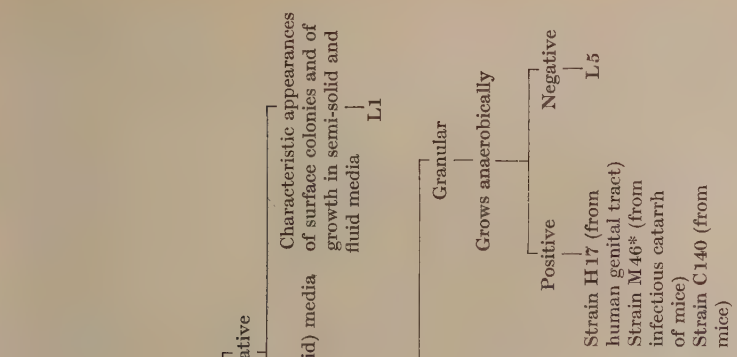
Varying amounts of the calcium salt of penicillin (Burroughs Wellcome and Co. product for veterinary use, containing 20,000 u. per tablet) were added to a series of bottles of the semi-solid medium without thallium acetate, one bottle being left free from penicillin as a control. Each bottle was inoculated similarly with a culture of strain B2 and well shaken; a loopful was then immediately subcultured on a plate. After incubation for 24 and 48 hr. further subcultures were made with a loop. Thus the bottle with the highest concentration of penicillin, in which definite multiplication of the organism occurred, was determined. The concentration was found by assay, using the cup method, to be 3000 u./ml. at the time of inoculation and 2200 u./ml. after incubation for 48 hr. Twice this amount of the preparation of penicillin did not dissolve completely and prevented multiplication of the organism.

The inactivation of the penicillin during incubation was due to its lability and was not caused by the action of the organism. This was shown by assaying the penicillin in a bottle of the medium, which, after the addition of the same amount of penicillin, was incubated for 48 hr. without having been inoculated. The fall in concentration was not significantly different from that in the bottle in which B2 was grown.

IDENTIFICATION OF MEMBERS OF THE PLEUROPNEUMONIA GROUP

In this investigation only stock strains, some maintained for many years in culture, were examined. It must be emphasized that there may be important differences in the properties of recently isolated strains. Moreover, only single strains of several organisms were examined. It is not certain that the properties are sufficiently constant to allow the identification of species within the pleuropneumonia group. The investigation, however, showed that most of the organisms studied could be identified by cultural properties. Only strains H17, M46 and C140 could not be differentiated from each other. A scheme whereby a culture might be identified is shown in Fig. 1; it is based on the ability to grow at room temperature and on a serum-free medium, and on the appearances of surface colonies and of cultures in semi-solid and fluid media. The examination of other properties could be used for confirmation.

In Table 6, in which the findings are summarized, the strains are divided into three groups: (1) the saprophytic group; (2) the L1 organism, isolated from a culture of a bacterium; (3) strains isolated independently from animals. The saprophytic group were classified by Sabin (1941*b*) in a different family from the other organisms of the pleuropneumonia group. No major difference was found between the two strains of sewage organism, but the one S strain from the bovine genital tract had slightly different fermentation reactions. Examination of a larger number of strains would be necessary to determine whether



* Did not grow anaerobically when first isolated.

Fig. 1. Identification of strains of pleuropneumonia-like organisms.

Table 6. *Summary of the properties of organisms of the pleuropneumonia group*

Organism	Growth at room temperature and on media *not* enriched with animal protein	Any characteristic appearances of culture	Growth in depths of semi-solid medium (i.e. not inhibited by anaerobic cultivation)	Growth in semi-solid and fluid media	Fermentation of carbohydrates	'Haemolysis'	Reduction of methylene blue
(A) Saprophytic species:							
Sewage A and B	Positive	None	Negative	Smooth	Positive	Positive	Positive
S strains from bovine genital tract	Positive	None	Negative	Finely granular	Positive	Positive	Positive
(B) Species isolated from cultures of bacteria:							
L1 (from *Streptobacillus moniliformis*)	Negative	Morphology and growth on solid and in semi-solid and fluid media characteristic	Negative	Large granular masses, usually heavily pigmented	Positive (including salicin)	Negative	Positive
(C) Species isolated from animals:							
Asterococcus bovis	Negative	None	Negative	Smooth	Positive	Positive (factor present in filtrates)	Positive
L3	Negative	Characteristic precipitate in media containing horse serum	Negative	Granular	Positive	Positive	Positive
L5	Negative	None	Negative	Granular	Positive	Positive	Positive
P strains from bovine genital tract	Negative	Characteristic precipitate in media containing horse serum	Positive	Smooth	Negative	Positive	Positive
L4	Negative	None	Positive	Smooth	Negative	Positive	Negative
Strain C140 from mice	Negative	None	Positive	Granular	Negative	Weakly positive†	Negative
Strain H17 from human genital tract	Negative	None	Positive	Granular	Negative	Negative	Negative
Strain M46 from infectious catarrh of mice	Negative	None	Positive*	Granular	Negative	Negative	Negative

* Negative when originally isolated.

† Broth cultures did not effect horse erythrocytes.

subdivision of the saprophytic group, according to cultural and biochemical properties, is possible. Organisms of the group are known to differ serologically (Laidlaw & Elford, 1936; Klieneberger, 1940; Edward, 1950).

The L1 organism differed so much from the others in its morphology and colonial appearances that it could be easily recognized. The other organisms are listed in Table 6 with those with greatest biological activity appearing first. Data regarding L3, L4 and L5 were obtained by examining only single strains. Although only one bovine P strain was examined completely, all the strains isolated produced the characteristic changes in horse-serum media; they gave smooth growths and were not inhibited by anaerobiosis. Haemolysis in blood agar plates was noted with all the strains tested.

Only one strain from the human genital tract was examined in detail. It is possible that more than one member of the pleuropneumonia group may inhabit the human genital tract, each differing in its properties, and possibly also in pathogenicity. A detailed examination of many strains would be of interest, cultural properties being compared with antigenic constitution.

DISCUSSION

The pleuropneumonia-like organisms share certain distinguishing properties. Their smallest viable units are minute spherical bodies which can pass bacteriological filters; ultrafiltration studies of certain species suggest that they measure *c.* 150–200 mμ. From these are developed larger bodies, which possess only a delicate cell membrane and are particularly fragile, thus partly explaining the peculiar pleomorphism of these organisms (Smith, Hillier & Mudd, 1948). In addition to reproduction by binary fission the large bodies multiply by multipolar germination. Pleuropneumonia-like organisms also produce characteristic colonies, due to growth into the medium. This probably depends upon a peculiarity in their metabolism. They are completely, or nearly completely, insensitive to the bacteriostatic action of penicillin.

The relationship of the pleuropneumonia group to the ordinary bacteria is complicated by the demonstration by Klieneberger (1936), Klieneberger-Nobel (1947) and Dienes (1949) that strains of pleuropneumonia-like organisms can be obtained from cultures of a number of bacteria. It now seems likely that these pleuropneumonia-like forms, or 'L forms', are variants of the bacteria. The L form differs from the original bacterium in its morphology, its smallest element being a particle which is filtrable, in its colonial appearance and in its resistance to penicillin. The last property facilitates its isolation in pure culture. The formation of a pleuropneumonia-like variant from *Bacterioides funduliformis* was studied under the electron microscope by Smith, Mudd & Hillier (1948). The bacillus itself had morphological similarities to a pleuropneumonia-like organism; certain of its forms were extremely fragile and large round bodies developed which multiplied by multipolar germination. Cells of the pleuropneumonia-like variant differed in being smaller. It would seem that the essential differences, which distinguish the pleuropneumonia-like from other organisms, are, apart from size, related to their metabolism rather than to their morphology.

Although pleuropneumonia-like variants of bacteria possess many characteristics typical of the pleuropneumonia group, many strains being stable with no tendency to revert to the bacillary form, it would seem desirable to classify them separately from the other members of the group. The L1 organism included in this investigation was significantly different from the other strains morphologically and culturally.

The investigation of pleuropneumonia-like organisms has hitherto been hindered by technical difficulties. It must therefore be emphasized that the examinations described can be easily carried out, provided that certain precautions are taken. In the first place it is necessary to use an adequately rich medium. Secondly, contamination, which is particularly liable to occur when plates are incubated for several days in a moist atmosphere, should be controlled by using suitable bacteriostatic substances. Thirdly, certain modifications to technique, such as the methods for inoculating and incubating media, are advisable.

I wish to express my thanks to Dr E. Klieneberger-Nobel, Dr W. J. Elford, Dr L. Dienes and Mr S. R. F. Bushby for supplying me with cultures.

REFERENCES

Beveridge, W. I. B. (1943). Isolation of pleuropneumonia-like organisms from the male urethra. *Med. J. Aust.* **2**, 479.

Bordet, J. (1910). La morphologie du microbe de la péripneumonie des bovides. *Ann. Inst. Pasteur*, **24**, 161.

Borrel, A., Dujardin-Beaumetz, Jeantet & Jouan (1910). Le microbe de la péripneumonie. *Ann. Inst. Pasteur*, **24**, 168.

Dienes, L. (1940). Cultivation of pleuropneumonia-like organisms from female genital organs. *Proc. Soc. exp. Biol., N.Y.*, **44**, 468.

Dienes, L. (1949). The development of *Proteus* cultures in the presence of Penicillin. *J. Bact.* **57**, 529.

Dienes, L., Ropes, M. W., Smith, W. E., Madoff, S. & Bauer, W. (1948). The role of pleuropneumonia-like organisms in genito-urinary and joint diseases. *New Engl. J. Med.* **238**, 509, 563.

Edward, D. G. ff. (1940). The occurrence in normal mice of pleuropneumonia-like organisms capable of causing pneumonia. *J. Path. Bact.* **50**, 409.

Edward, D. G. ff. (1947*a*). A selective medium for pleuropneumonia-like organisms. *J. gen. Microbiol.* **1**, 238.

Edward, D. G. ff. (1947*b*). Catarrh of the upper respiratory tract in mice and its association with pleuropneumonia-like organisms. *J. Path. Bact.* **59**, 209.

Edward, D. G. ff. (1950). An investigation of pleuropneumonia-like organisms isolated from the bovine genital tract. *J. gen. Microbiol.* **4**, 4.

Edward, D. G. ff., Hancock, J. L. & Hignett, S. L. (1947). Isolation of pleuropneumonia-like organisms from the bovine genital tract. *Vet. Rec.* **59**, 329.

Findlay, G. M., Klieneberger, E., MacCallum, F. O. & Mackenzie, R. D. (1938). Rolling disease. New syndrome in mice associated with a pleuropneumonia-like organism. *Lancet*, ii, 1511.

Holmes, B. E. & Pirie, A. (1932). Growth and metabolism of the bovine pleuropneumonia virus. *Brit. J. exp. Path.* **13**, 364.

Klieneberger, E. (1936). Further studies on *Streptobacillus moniliformis* and its symbiont. *J. Path. Bact.* **42**, 587.

Klieneberger, E. (1938). Pleuropneumonia-like organisms of diverse provenance; some results of an enquiry into methods of differentiation. *J. Hyg., Camb.*, **38**, 458.

Klieneberger, E. (1939). Studies on pleuropneumonia-like organisms: the L4 organism as the cause of Woglom's 'pyogenic virus'. *J. Hyg., Camb.*, **39**, 260.

Klieneberger, E. (1940). The pleuropneumonia-like organisms: further comparative studies and a descriptive account of recently discovered types. *J. Hyg., Camb.*, **40**, 204.

Klieneberger-Nobel, E. (1947). Isolation and maintenance of an L1-like culture from *Fusiformis necrophorus* (Syn. *Bact. funduliforme. Bacteroides funduliformis*). *J. Hyg., Camb.*, **45**, 407.

Klieneberger, E. & Steabben, D. B. (1940). On the association of the pleuropneumonia-like organism L3 with bronchiectatic lesions in rats. *J. Hyg., Camb.*, **40**, 223.

Laidlaw, P. P. & Elford, W. J. (1936). A new group of filtrable organisms. *Proc. Roy. Soc.* B, **120**, 292.

Nocard & Roux (1898). Le microbe de la péripneumonie. *Ann. Inst. Pasteur*, **12**, 240.

Preston, W. S. (1942). Arthritis in rats caused by pleuropneumonia-like micro-organisms and the relationship of similar organisms to human rheumatism. *J. infect. Dis.* **70**, 180.

Sabin, A. B. (1939). Mice as carriers of pathogenic pleuropneumonia-like micro-organisms. *Science*, **90**, 18.

Sabin, A. B. (1941*a*). The filtrable micro-organisms of the pleuropneumonia group. *Bact. Rev.* **5**, 1.

Sabin, A. B. (1941*b*). The filtrable micro-organisms of the pleuropneumonia group. (Appendix on classification and nomenclature.) *Bact. Rev.* **5**, 331.

Seiffert, G. (1937). Ueber das Vorkommen filtrabler Mikroorganismen in der Natur und ihre Züchtbarkeit. *Zbl. Bakt.* (1 Abt., Orig.) **139**, 337.

Smith, W. E., Hillier, J. & Mudd, S. (1948). Electron micrograph studies of two strains of pleuropneumonia-like (L) organisms of human derivation. *J. Bact.* **56**, 589.

Smith, W. E., Mudd, S. & Hillier, J. (1948). L type variation and bacterial reproduction by large bodies as seen in electron micrographic studies of *Bacteroides funduliformis*. *J. Bact.* **56**, 603.

Tang, F. F., Wei, H., McWhirter, D. L. & Edgar, J. (1935). An investigation of the causal agent of bovine pleuropneumonia. *J. Path. Bact.* **40**, 391.

Topley, W. W. C. & Wilson, G. S. (1946). *The principles of bacteriology and immunity*, 3rd ed. Revised by Wilson, G. S. & Miles, A. A. London.

Warren, J. (1942). Observations on some biological characteristics of the pleuropneumonia group. *J. Bact.* **43**, 211.

(*Received* 3 *November* 1949)

Factors Affecting the Germination of Thick Suspensions of *Bacillus subtilis* Spores in L-Alanine Solution

By JOAN F. POWELL

Microbiological Research Department (Ministry of Supply), Porton, Wiltshire

SUMMARY: The rate of germination (defined as loss of heat-resistance accompanied by change in staining properties under specified conditions, with maintenance of viability) of thick suspensions of *Bacillus subtilis* spores in phosphate-buffered L-alanine solution increased with the time from harvesting. The maximum rate of germination was reached after about 20 days' storage in water at 20°. This effect could be retarded, but not reversed, by storage at low temperatures. The rate of germination may be temporarily accelerated by heat treatment. Germination was considerably retarded after treatment with mercuric chloride, and was completely inhibited by 8-hydroxyquinoline (oxine) and by 2:3-dimercaptopropanol (BAL) at 10 mM concentration. The latter effect was partially reversed by the addition of metals.

Using dilute spore suspensions of *Bacillus subtilis* (10^4 spores/ml.) Hills (1950) found that 80 % loss of heat-resistance occurred during 30 min. incubation at 35° in 0·5 mM L-alanine buffered with 33 mM phosphate at pH 7·3. The present report concerns germination in thicker suspensions, i.e. 5×10^9 spores/ml., with a view to the study of the biochemical changes which accompany loss of heat-resistance in the above simple medium.

EXPERIMENTAL

A laboratory strain of *B. subtilis* was used throughout. Suspensions containing at least 95 % spores were obtained by growth on CCY agar (Gladstone & Fildes, 1940) at 37° for 12 days. The spores were washed five times with distilled water after harvesting. Their age was measured from the day of reaping, and storage was at room temperature (20°) unless otherwise stated. No heat treatment was given after harvesting except that specifically stated in an experiment.

The percentage germination was at first determined by plate counts on peptone agar before and after heating at 60° for 15 min. (Hills, 1950). Later, it was found that when films of the L-alanine-treated spores were made, fixed and stained with hot carbol fuchsin and methylene blue, most of the cells appeared elongated and stained uniformly bluish purple or showed a central blue-staining body (Pl. 1). These were readily distinguishable from the unchanged spores, and their percentage occurrence could be determined by direct counting.

The details of the staining procedure were as follows. Films were treated for 5 min. with hot carbol fuchsin, then washed thoroughly with hot water. Nigrosin (1 %) was then poured on, and left for 2 min. After washing in cold water, the slide was stained with methylene blue for 2 min. It was found important to avoid overstaining with carbol fuchsin since differentiation between the two forms was then very poor. Twenty-two samples of partially germinated suspensions were stained and counted in this way, and the proportion of the blue-staining forms determined. The value obtained agreed very well with that

for non-heat-resisting cells, determined by the plating method in the same samples (Fig. 1). The total number of cells counted in both methods was about 600, and the blue-staining or non-heat-resisting cell count ranged from 300 to 500, so that the standard error of counting was 4–6 %. It thus appears that loss of heat-resistance can be estimated equally well by counting the cells which have undergone the above change in staining properties, which may be connected with a sudden increase of cell permeability. The plating method was therefore abandoned in favour of the less laborious staining technique, except in those cases where it was necessary to determine the viability of the germinated cells.

It was also observed that the turbidity of a spore suspension markedly decreased on incubation with L-alanine. This phenomenon was obviously due to the decrease in refractive index and therefore in the amount of light scattered by the spore during germination. Measurements of percentage transmission of germinating suspensions were made at 610 mμ. in a Coleman spectrophotometer. Using this simple turbimetric method at 37°, many readings may be made at short intervals, and the effect of storing and pre-heating of spores very clearly demonstrated (see below).

RESULTS

The germination rate of thicker spore suspensions, i.e. 10^9 spores/ml. in 0·5 mM L-alanine at 35° was slower and less complete than that of dilute suspensions. It could be increased by raising the L-alanine concentration to 5 mM and adding 50 mM glucose, the mixture being buffered by 33 mM phosphate at pH 7·3 as before. Under these conditions, 80–90 % germination occurred in 30 min. and plate counts showed that even after 2–3 hr. incubation, all the cells remained viable.

The effect of substituting acetate and bicarbonate buffer for phosphate is shown in Table 1. The buffer-capacity of 33 mM acetate at neutrality is low, so that here the L-alanine concentration was decreased to 0·5 mM. The germination rate in this mixture without glucose was at least equal to, or possibly greater than, that in phosphate buffer of the same pH, and all the cells were viable at the end of the experiment. Germination proceeded as well in bicarbonate as in phosphate buffer at pH 8·1. In this case, glucose was present and the L-alanine concentration was 5 mM, so that germination was more rapid and extensive than in the acetate experiment at pH 6·8. The difference in percentage germination between the phosphate-bicarbonate and the phosphate-acetate comparison experiments cannot be ascribed to the difference in pH, for the germination of a 10^4 spores/ml. suspension in 0·5 mM L-alanine measured at 1·5 hr. in phosphate buffer of pH ranging between 6·0 and 8·0, did not vary appreciably between pH 6·5 and 8·0.

The germination of freshly harvested spores was very slow and incomplete even in 5 mM L-alanine and glucose. On storing the aqueous spore-suspension at room temperature the germination rate gradually increased to a maximum at about 20 days (Fig. 2). For example, a 3-day-old suspension showed only

Table 1. *Comparison of germination of spores of* Bacillus subtilis *on incubation in acetate-, bicarbonate- or phosphate-buffered* L-*alanine solutions*

(Temperature of incubation 35°; samples removed at intervals.)

Composition of incubation solution	pH	Period of incubation (min.)	Film counts total/germinated	Germination (%)
33 mM phosphate	6·86	10	689/260	38
0·5 mM L-alanine		20	716/387	59
		40	665/384	58
		80	738/446	60
33 mM acetate	6·81	10	627/262	42
0·5 mM L-alanine		20	620/402	65
		40	585/433	73
		80	598/429	72
33 mM phosphate	8·10	18	701/566	81
5 mM L-alanine		30	706/635	90
50 mM glucose		60	678/629	93
		100	657/615	94
33 mM bicarbonate	8·10	18	750/559	75
5 mM L-alanine		30	657/506	77
50 mM glucose		60	624/493	78
		100	636/529	83

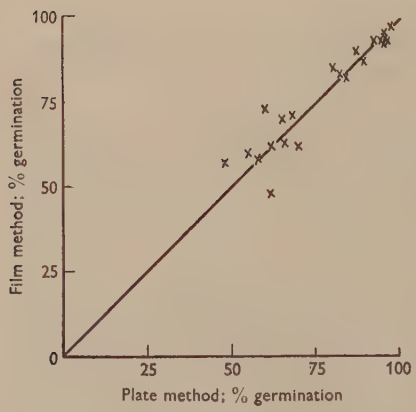


Fig. 1

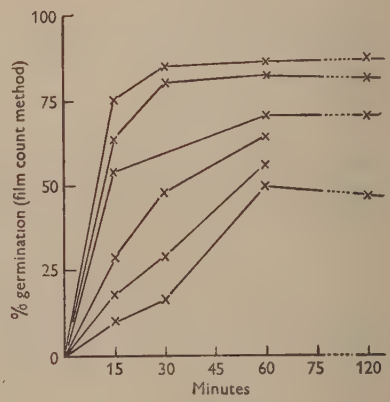


Fig. 2

Fig. 1. Comparison of percentage germination in twenty-two samples measured by the filming and plating techniques.

Fig. 2. Effect of increasing periods of storage (3 to 25 days at 20°) on the germination of *B. subtilis* spores in 33 mM phosphate, 50 mM glucose and 5 mM L-alanine at pH 7·3 and 35°.

10 % germination after 15 min. incubation, but after 25 days' storage at 20°, 75 % germination occurred in 15 min.

This spontaneous increase in germination rate during storage, conveniently referred to as ageing, was slower at low temperatures. Table 2 shows the germination rate of two samples of the same suspension, one of which had been stored at 20°, the other at 4° for 31 days, compared with the germination rate

of the same suspension when freshly reaped. Storage of aged cells at low temperature did not, however, result in a decreased germination rate. Neither was it affected by a 3-fold washing of an aged suspension (25 days at 20°). Ageing was therefore not due to breakdown of some spores with loss of diffusible constituents which stimulated the germination of the remainder.

Table 2. *The effect of storage of* Bacillus subtilis *spores in water at* 20° *and* 4°

(Germination was examined by incubation at 35° in phosphate buffer (33 mM; pH 7·3) containing L-alanine (5 mM) and glucose (50 mM). Samples were examined after different periods of incubation.)

Treatment of spore suspension	Period of incubation (min.)	Film counts total/germinated	Germination (%)
Control before storage	15	—	10
	30	519/87	15
	60	752/373	50
	120	742/341	46
Stored for 31 days in water at 20°	15	690/497	72
	30	626/515	82
	60	656/564	86
	120	703/604	86
Stored for 31 days in water at 4°	15	633/114	18
	30	739/283	38
	60	543/321	61
	120	596/406	68

In order to determine whether spores would age in the above sense, on prolonged contact with the CCY agar on which they had been grown, the germination of a 3-day-old suspension, reaped after 30 days' incubation, was measured by the staining method; 5, 30 and 72 % germination occurred at 15, 30 and 60 min. respectively, which is a performance characteristic of a still 'young' suspension.

The effect of ageing on germination was also demonstrated by turbidity measurements during incubation with L-alanine. Fig. 3 shows that the rate of increase of light transmission was very much greater in the case of aged than of freshly reaped spores.

A temporary increase in the rate of germination was produced by heating an aqueous spore suspension at 60° before its addition to the buffered alanine. Table 3 compares the effect of pre-heating a freshly reaped, i.e. a 3-day-old spore suspension for 15 min., and for 3·5 hr. In Fig. 4, the result of a similar experiment with a 7-day-old spore suspension is shown, the turbidity of the suspension being measured at short intervals during germination. Here, the times of pre-heating were 30 min. and 3·5 hr. The effect of heat treatment was almost completely lost in about 48 hr. (Table 3), so that the heat-activation process seems not to involve the same mechanisms as the ageing process.

In order to test for germination stimulants produced in spores during heating, a 10^9 spores/ml. suspension, heated for 2 hr. at 60°, was broken up with glass beads in a Mickle tissue disintegrator (Mickle, 1948). After centrifuging and Seitz filtration, 1 ml. of the extract was added to 9 ml. of a 10^4/ml. spore sus-

Table 3. *Heat activation of germination of* Bacillus subtilis *spores*

(The spore suspensions were treated as shown. The degree of germination after different periods of incubation as 35° in phosphate buffer (33 mM; pH 7·3) containing L-alanine (5 mM) and glucose (50 mM) was then measured.)

Treatment of spore suspension	Period of incubation (min.)	Film counts total/germinated	Germination (%)
Control (age 3 days)	15	667/70	11
	30	623/179	29
	63	643/266	41
	100	603/252	42
15 min. pre-heated at 60°	15	592/67	13
	30	622/339	38
	63	612/330	54
	100	625/333	53
3·5 hr. pre-heated	15	623/317	49
	30	662/440	66
	63	695/498	72
	100	650/474	73
48 hr. after 3·5 hr. pre-heating	15	525/89	17
	30	577/222	38
	63	590/352	60
	100	570/312	55

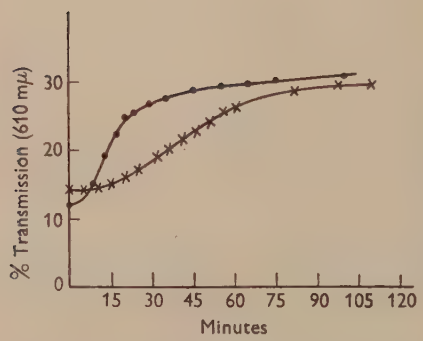


Fig. 3

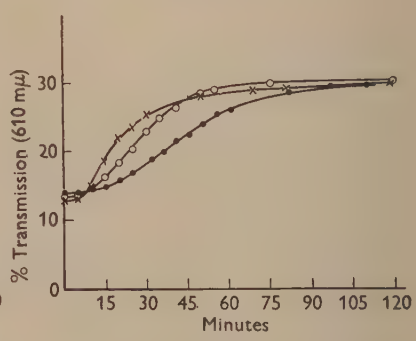


Fig. 4

Fig. 3. Comparison of the germination rates of a 7-day-old (×——×) and a 38-day-old (●——●) spore suspension of *B. subtilis* (storage at 20°) in 33 mM phosphate, 50 mM glucose and 5 mM L-alanine at pH 7·3 and 35°. Measurements of percentage transmission made at 610 mμ.

Fig. 4. The effect of pre-heating for 30 min. (○——○) and for 3·5 hr. (×——×) on the germination of *B. subtilis* spores in 33 mM phosphate, 50 mM glucose and 5 mM L-alanine at pH 7·3 and 35°. Control unheated spores, ●——●. Measurements of percentage transmission made at 610 mμ.

pension in phosphate buffer. Thus there was available to each spore an amount of extract equivalent to 10^4 heated spores. Even after incubation for 2 hr. at 35°, there was no evidence of germination, as measured by the plating technique. Similarly, the germination of a 10^4/ml. spore suspension after 1 hr. incubation in 0·5 mM L-alanine was not stimulated by the addition of pre-heated spore

extract. In the latter experiment there was available to each spore an amount of extract equivalent to 3×10^4 heated spores.

Effect of certain enzyme inhibitors

A few experiments were done to determine the effects of certain enzyme inhibitors on the germination process in L-alanine. Keilin & Hartree (1947) showed that the germination of *B. subtilis* spores in glucose yeast extract medium was completely inhibited by a 20 μM concentration of 8-hydroxyquinoline (oxine). The inhibition was removed by washing and re-suspending the spores in fresh medium. These authors generally used the sudden increase in rate of oxygen uptake accompanying proliferation as an index of germination; in the oxine experiment, however, the inhibition was 'confirmed by microscopic examination'. The resting metabolism of both the spore and the vegetative cell was found to be considerably less sensitive to oxine, a concentration of 1 mM being required to decrease the rate of oxygen uptake by 30 %. The germination of *B. subtilis* spores in the present investigation, as measured by both the staining and the plating techniques, was unaffected by 1 mM oxine and all the cells remained viable. In the presence of 10 mM oxine germination was completely inhibited. In these experiments the spores in 10^9/ml. suspension were first incubated with glucose, buffer and oxine for 20 min.; the L-alanine was then added, and germination measured after a further 30 and 60 min. incubation.

Using the same conditions as in the above experiment, similar results were obtained with 2:3-dimercaptopropanol (BAL). This compound inhibits certain metal-activated enzyme systems at concentrations of 1–10 mM (Webb & van Heyningen, 1947; Barron, Miller & Meyer, 1947). Concentrations of BAL up to 2 mM did not appreciably diminish spore germination as indicated by staining. On increasing the BAL concentration, inhibition became noticeable at 4 mM, and complete at 10 mM. A partial reversal of the inhibition was obtained by the addition of soluble salts of zinc, magnesium, copper and iron. The germination thus observed after 30 and 60 min. incubation varied between 10 and 30 %. It was not possible to make satisfactory counts with these suspensions since even after treating the slide with dilute acid to remove precipitated BAL-metal complex, the cells remained in clumps.

To test whether the viability of spores was affected by concentrations of BAL which inhibited germination, a 10^9 spores/ml. suspension was incubated with 10 mM BAL in L-alanine glucose buffer mixture for 30 min. The suspension was also treated with 10 mM BAL alone for 30 min. Both samples were then diluted $1/10^6$ and plated. In the former case the viable count had fallen by 20 %, but remained unaltered in the latter.

The effect of pre-treatment of spores with mercuric chloride before incubation with L-alanine was also studied. A 10^9 spores/ml. suspension in 5 % mercuric chloride was allowed to stand at room temperature for 40 min. after which the mercuric chloride solution was removed, and the spores washed four times with water. No visible colonies were produced by plating the treated suspension on peptone agar. When, however, the 'non-viable' suspension was

incubated with L-alanine and glucose, within 60 min. 80 % of the spores showed the change in staining properties associated with germination. The process was, however, markedly slower than that in a control suspension (Table 4).

With regard to the viability of mercuric chloride treated spores, 80 % of the original viable count was reached when the spores were plated on peptone agar to which 0·1 % sodium thiolacetate had been added. The reviving effect of 0·1 % BAL was also tested, but found to be less than that of thiolacetate. This may be due to the decomposition of BAL during the drying of the plates; the

Table 4. *Germination of mercury-treated* Bacillus subtilis *spores*

(Spores (10^9/ml.) in 5 % mercuric chloride for 40 min. at room temperature. Spores then centrifuged out and washed four times with 20 ml. lots of water. Effect on germination examined by incubation at 35° in phosphate buffer (33 mM; pH 7·3) containing L-alanine (5 mM) and glucose (50 mM), samples being examined after the periods shown.)

Treatment of spore suspension	Period of incubation in buffer (min.)	Film counts total/germinated	Germination (%)
Control	15	735/461	63
	30	579/463	80
	60	664/545	82
Mercury treated	15	—	10
	30	671/339	50
	60	747/594	80

medium became heavily clouded with sulphur. A mercury-treated suspension which had been incubated with L-alanine for 1 hr., and showed 80 % change in staining properties, was also tested for viability on thiolacetate agar. Here, only 15 % revival was obtained. This was slightly increased, however, by including 0·1 % thiolacetate in the final dilution, which was allowed to stand for 1 hr. before plating. When BAL was substituted for thiolacetate in the plated dilution, 50 % revival was obtained.

DISCUSSION

Curran & Evans (1945) showed that pre-heating stimulated the germination of certain thermo-tolerant bacteria, e.g. *Bacillus coagulans* and *B. calidolactis*. A similar effect was observed by Goddard (1939) in the germination of ascospores of *Neurospora tetrasperma*. Here it was found that the heated spores became deactivated on storage for a few hours under anaerobic conditions, or in the presence of cyanide, but could be reactivated by further heat-treatment. With regard to the mechanism of heat-activation, it is reasonable to apply the suggestion made by Goddard (1939), namely, that the rate of spore germination may be at least partly determined by the concentration of a stimulatory compound in the spore, and that the supply of this compound is limited by a heat-activated reaction.

The irreversible ageing process is more difficult to explain. Spores which remain in contact with the solid medium for long periods germinate far less

readily in L-alanine solution than do those which have been stored in watery suspensions for the same period. It is possible that the newly formed spore contains substances which antagonize its germination and that these substances are lost when the spore is harvested, but not when it remains in contact with the medium in which it has grown and which may now contain an unfavourable balance of nutrients as well as products of metabolism. Since the ageing effect can be retarded by storage at low temperatures, it may be suggested that the inhibitory substance is lost by a heat-activated adsorption process (Taylor, 1932).

It is interesting to note that Schwann (1924) found that in the case of *Bacillus anthracis*, the percentage germination as directly observed on nutrient agar decreased from 95 % in young spores, i.e. up to 7 days old, to 55 % after storage for a year. In these experiments, however, germination was taken as the appearance of the first cell division, and the ageing process took place under drying conditions, so that these results cannot be directly compared with those reported here.

Spore germination in L-alanine is comparatively insensitive to oxine, a concentration of 10 mM being required for complete inhibition of the change in heat resistance and staining properties. This concentration is high compared with that found by Keilin & Hartree for *B. subtilis* spores in yeast extract medium. These authors, however, included growth and proliferation in their criterion of germination, and it is likely that the two latter processes are more sensitive to oxine than is the initial change involving loss of heat-resistance. The concentration found in the present work to be necessary for inhibition of germination is of the same order as that found by Keilin & Hartree to inhibit the resting metabolism of the spore, i.e. the respiration in glucose and phosphate buffer.

The effect of BAL on spore germination is very similar to that of oxine. Both these compounds readily form complexes with metals and may be expected to inhibit those enzyme systems which are metal-activated. The reversal of BAL inhibition by addition of excess of metal salts has not been satisfactorily assessed.

The behaviour of mercury-treated spores with L-alanine is surprising. Since a high proportion of spores can be revived after mercury treatment, it seems fairly certain that the change in staining properties is a genuine vital process, and not due to damage to the cell by mercury. The results suggest that the change in heat-resistance can occur in cells which are incapable of growth and division; this is also suggested by the experiments with oxine. It may, however, be argued that the mercury ion does not, in fact, enter the spore, but remains attached externally to the spore coat and exerts its bacteriostatic effect during the next stage of development. Germination is appreciably slowed by pretreatment with mercury salt, but it cannot be proved that the phenomenon is due to entry of mercury and not to a change in permeability of the spore-wall caused by combination with mercury.

I wish to thank Dr D. W. Henderson and Mr G. M. Hills for their suggestions and criticism and Miss N. Harris for the photomicrograph. The work was carried out with the technical assistance of Miss B. Rice. Permission to publish has been granted by the Chief Scientist, Ministry of Supply.

REFERENCES

Barron, E. S. G., Miller, Z. B. & Meyer, J. (1947). The effect of 2:3-dimercaptopropanol on the activity of enzymes and on the metabolism of tissues. *Biochem. J.* **41**, 78.

Curran, H. R. & Evans, F. R. (1945). Heat activation inducing germination in the spores of thermotolerant and thermophilic aerobic bacteria. *J. Bact.* **49**, 335.

Gladstone, G. P. & Fildes, P. (1940). A simple culture medium for general use without meat extract or peptone. *Brit. J. exp. Path.* **21**, 161.

Goddard, D. R. (1939). The reversible heat activation of respiration in *Neurospora*. *Cold Spr. Harb. Sym. quant. Biol.* **7**, 362.

Hills, G. M. (1950). Chemical factors in the germination of spore-bearing aerobes: observations on the influence of species, strain, and conditions of growth. *J. gen. Microbiol.* **4**, 38.

Keilin, D. & Hartree, E. F. (1947). A comparative study of spores and vegetative forms of *Bacillus subtilis*. *Antonie v. Leeuwenhoek, J. Microbiol. Serol.* **12**, 115.

Mickle, H. (1948). Tissue disintegrator. *J. R. micr. Soc.* **68**, 10.

Schwann, M. B. R. (1924). On the germination period and mortality of the spores of *Bacillus anthracis*. *J. Path. Bact.* **27**, 130.

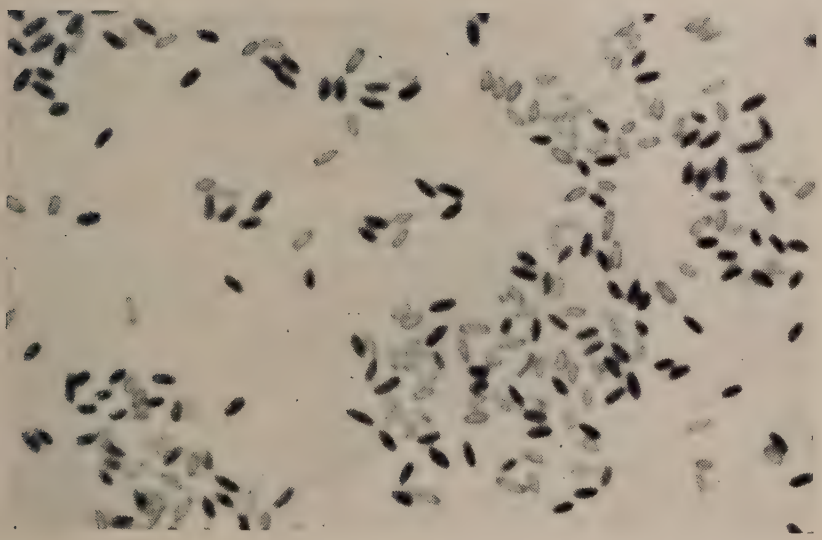
Taylor, H. S. (1932). The adsorption of gases by solids. *Trans. Faraday Soc.* **28**, 131.

Webb, E. C. & van Heyningen, R. (1947). The action of British anti-lewisite on enzyme systems. *Biochem. J.* **41**, 74.

EXPLANATION OF PLATE

Bacillus subtilis spores showing densely staining forms after incubation at 35° for 15 min. in 33 mM phosphate, 50 mM glucose and 5 mM L-alanine and stained with carbol fuchsin and methylene blue. Magnification, ×2850.

(*Received* 3 *November* 1949)



J. F. Powell—Factors affecting the germination of thick suspensions of *Bacillus subtilis* spores in L-alanine solution. Plate 1

Colony Counts on Strips of Agar in Tubes

By ANNA C. STIRLING, MARION K. STEVENS and D. N. LAWLEY

School of Agriculture, Edinburgh

SUMMARY: For viable counting, 0·1 ml. volumes of bacterial suspension were incorporated in 1·5–2 ml. nutrient agar, which after mixing was allowed to solidify in an almost horizontal test-tube so as to produce a 3–4 in. agar strip. After incubation in the vertical position, the counts of the strips gave estimates of a bacterial population which did not differ significantly from that obtained from plates inoculated with 1 ml. quantities.

The study of bacterial populations requires a simple method for determining the viable count. The method most often used is the plate count, but with a large number of plates this method becomes rather cumbersome and expensive in equipment, medium and laboratory labour. Modifications of the plating method have been suggested from time to time with a view to reducing the amount of materials and labour involved without impairing the accuracy of the count (Esmarch, 1886; Miles & Misra, 1938). In an endeavour to find a simple method by which colony counts might be made, inoculation of small quantities of agar in test-tubes was tried.

EXPERIMENTAL

Preliminary trials were carried out to determine the most suitable quantity and concentration of agar to use in each tube, and the best methods of mixing and of forming the agar strips. In the technique finally adopted $6 \times \frac{5}{8}$ in. test-tubes were used, each containing 1·5–2 ml. of 2 % nutrient agar. The agar was melted and held at 45° for $\frac{1}{2}$–1 hr. before it was inoculated. This allowed condensation water to run down into the agar, thus lessening the amount of moisture appearing at the foot of the tubes during incubation. Much moisture encouraged spreading growth, especially when the count was high.

A 1 ml. pipette graduated in 0·01 ml. was used to deliver 0·1 ml. of inoculum into each tube.

The agar and inoculum were mixed by rotating the tube five times, and the tube was then laid on the bench with the plugged end slightly raised so as to produce a layer of agar 3–4 in. long. When necessary the tubes were slightly tilted when being laid down to ensure that the agar flows evenly over the glass. The colonies that developed were counted with greater ease when the strip of agar was of good length. The agar solidified rapidly and when the tubes were cool they were set upright in racks and placed in the incubator.

Experiments were carried out to provide material for a statistical analysis, using a suspension of the spores of *Bacillus megatherium* which was stable and gave reproducible results.

Exp. 1. This experiment was designed to compare the counts obtained in tubes with those obtained by the standard plating method in which 1 ml.

quantities of spore suspension were mixed with approximately 10 ml. of nutrient agar in Petri dishes.

Six workers each inoculated 120 tubes and ten plates; each worker used one pipette for the inoculation of both tubes and plates. In order to detect any differences due to pipetting 0·1 ml. quantities the tube inoculations were divided into three groups:

(*a*) Forty tubes were inoculated with consecutive 0·1 ml. amounts.

(*b*) Forty tubes were inoculated with alternate 0·1 ml. amounts, i.e. 1st, 3rd, 5th, 7th and 9th 0·1 ml. amounts.

(*c*) Forty tubes were inoculated with the 1st and 6th amount of 0·1 ml. from each pipetteful. In (*b*) and (*c*) the unused quantities were discarded into a sterile tube.

Table 1. *Mean colony counts and standard errors obtained by plate and strip counts*

Worker ...	A	B	C	D	E	F	All workers
Mean count in 10 plates	302·6 ±7·5	288·8 ±4·7	295·5 ±9·6	302·8 ±8·7	296·4 ±5·4	312·7 ±5·2	299·8 ±2·9
Mean count in 120 tubes (×10)	312·0 ±5·4	299·2 ±5·7	298·2 ±5·0	309·6 ±6·7	296·7 ±5·1	290·9 ±4·8	301·1 ±2·2

The mean count for all the plates (Table 1) (1 ml. inoculum) was 299·8 ± 2·9, and that for tubes (×10, since in this case the inoculum was 0·1 ml.) was 301·1 ± 2·2. These counts do not differ significantly.

Table 2. *Analysis of variance of strip counts of experiment recorded in Table* 1

Method of inoculation	Factors	Degrees of freedom	Sum of squares	Mean square	Variance ratio
Consecutive 0·1 ml. amounts	Position of 0·1 ml. amounts in the pipette (*P*)	9	880·0	97·8	2·77*
	Interaction of workers and positions (*W* × *P*)	45	1,504·6	33·4	0·95
	Pipettefuls (*Pp*)	18	820·3	45·6	1·29
Alternate 0·1 ml. amounts	*P*	4	188·9	47·2	1·34
	W × *P*	20	855·3	42·8	1·21
	Pp	42	900·2	21·4	0·61
1st and 6th 0·1 ml. amounts	*P*	1	82·8	82·8	2·35
	W × *P*	5	348·0	69·6	1·97
	Pp	114	4,395·0	38·6	1·09
Combined results	Workers (*W*)	5	437·0	87·4	2·48†
	Methods (*M*)	2	137·1	68·5	1·94
	Interaction (*W* × *M*)	10	401·7	40·2	1·14
	Error	433	15,277·6	35·3	—
	Total	708	26,228·5	—	—

* Significant at the 1 % level. † Significant at the 5 % level.

The results of the tube counts were subjected to an analysis of variance (Table 2) to ascertain the accuracy of the technique. The variance ratio calculated from the combined results of the six workers indicated a significant difference between workers at the 5 % level, which may have been due to inexperience with the method. There was no significant difference between the various pipettefuls measured or between the three methods of inoculation, but counts from consecutive 0·1 ml. inocula differed significantly at the 1 % level when the results for all workers were combined, although not when the results were examined separately for each worker. The discrepancy with the consecutive 0·1 ml. amounts appeared to be due mainly to the last 0·1 ml. delivered by each pipette, which in most cases gave a higher mean count than the remainder, as is shown in Table 3. This higher count may have been due to the slowness of

Table 3. *Mean counts of consecutive* 0·1 *ml. amounts delivered by* 1 *ml. pipettes*

Successive 0·1 ml. amount ...	1	2	3	4	5	6	7	8	9	10
Mean count	31·25	28·92	29·79	29·79	29·58	28·17	26·46	30·08	27·88	33·88

delivery of the pipette which was obvious with the last 0·1 ml. volume, or to the difficulty in seeing the graduation mark, which came within the test-tube. This source of error could be avoided by not using the last 0·1 ml. quantity. The seventh 0·1 ml. quantity appears to be somewhat smaller than the others, though the divergence is not so marked as with the tenth. A check of the pipettes by weighing the quantities delivered did not reveal any peculiarity of the seventh 0·1 ml. volume.

The value of χ^2 for tube counts, calculated by dividing the error sum of squares of the analysis of variance (Table 2) by the mean count for all tubes, was 507·4 with 433 degrees of freedom. This is significantly large at the 1 % level. Further examination revealed, however, that this high value was almost entirely due to the variable counts obtained by one worker, for which there may have been some special reason. When the results for this worker were omitted the value of χ^2 was 388·4, which with 363 degrees of freedom is not significant. There is thus no indication that the counts obtained from tubes deviated significantly from the expected Poisson distribution. A given number of tubes did not, however, give as reliable a result as the same number of plates, the coefficient of variation for plates being 7·3 % and for tubes 19·9 %. This was thought to be due to the number of colonies counted in plates having been larger than in tubes, so a second experiment was designed to show the influence of colony numbers.

Exp. 2. In this experiment two dilutions of spore suspension were used, one ten times as concentrated as the other. Two workers each inoculated twelve plates with 1 ml. quantities of the higher dilution and 120 tubes with each dilution, using consecutive 0·1 ml. amounts of inoculum. The number of colonies developing on a plate should therefore be the same as the number developing in a tube inoculated with the lower dilution and thus permit a direct comparison of the two methods.

Table 4. *Mean counts in plates and strips, and the observed and expected coefficients of variation* (*Exp.* 2)

	Mean count		Coefficient of variation	
			Observed (%)	Expected (%)
12 plates	Worker 1	75·25 ± 2·46	11·3	11·5
12 plates	Worker 2	76·25 ± 2·37	10·8	11·4
Low dilution				
120 tubes	Worker 1	74·99 ± 0·89	13·0	11·6
120 tubes	Worker 2	72·08 ± 0·75	11·3	11·8
High dilution				
120 tubes	Worker 1	7·77 ± 0·24	33·3	35·9
120 tubes	Worker 2	7·62 ± 0·24	33·8	36·2

For the same mean count there was no significant difference between the plate method and the tube method (Table 4). The means given by the two workers were in good agreement. The observed coefficients of variation agree well with their expected values. In only one case, namely that of worker 1 with the 'low dilution' tubes, was the observed coefficient of variation greater than or appreciably different from expectation. An analysis of variance showed no significant difference between counts from different 0·1 ml. quantities or from different pipettefuls.

This experiment indicates that one tube inoculated with a 0·1 ml. quantity can replace one plate inoculated with a 1 ml. quantity, provided the resulting mean colony counts lie at the same level.

DISCUSSION

The inoculation of agar in tubes in the manner described above is simple, and enables many replicate counts to be made with little expenditure of time, labour or materials. The method has advantages over roll-tube methods in that it permits easier counting and decreases the amount of manipulation. It also compares favourably with surface-counting methods (Snyder, 1947; Crone, 1948) where plates must be prepared beforehand and dried, and where considerable amounts of medium are required. There are, however, several points which require consideration.

The concentration of agar used. The accuracy of the count is diminished when the agar slips within the tubes during incubation. This can be avoided by using a suitable concentration of agar. With the several media and incubation temperatures that have been used, 2 % agar gave a sufficiently large margin of safety without inhibiting bacterial growth. This concentration might have to be altered to suit special conditions.

The glassware. Since the quantity of agar in each test-tube is small, the tubes must be perfectly clean; any trace of an antibacterial substance might diminish the count or delay the appearance of colonies.

The inoculation. It is generally accepted that the sampling error is apt to become very large when volumes less than 1 ml. are measured, but our results

suggest that this need not be so. With normal care, the error introduced by the measurement of 0·1 ml. from a 1 ml. pipette is cancelled by other errors that are inherent in any plating method. Snyder (1947) found that 0·1 ml. delivered from a capillary dropping pipette was more accurate than 0·1 ml. from a 1 ml. pipette, but concluded that there was no appreciable contribution from the pipetting error unless the total error of the method was very small. One method recommended by Snyder was studied in more detail and analysed statistically by Crone (1948). Counts obtained by spreading 0·1 ml. inocula, measured by a capillary pipette or a 1 ml. graduated pipette, over the surface of previously prepared plates, were shown to conform within the limits of experimental error to a Poisson distribution, and the total count from all the plates agreed well with that obtained from a corresponding number of roll-tube cultures. No large error therefore appears to have been introduced in the measurement of 0·1 ml. inocula. Miles & Misra (1938) used a capillary pipette for the seeding of 0·02 ml. drops on agar plates. Greater accuracy of measurement of the inoculum could undoubtedly be achieved by the use of calibrated dropping pipettes, but for the inoculation of tubes the 1 ml. pipette is easier to handle, and provided that the last 0·1 ml. quantity is not used, there seems little disadvantage in the use of the 1 ml. pipette. Pipettes with good points and a suitable rate of delivery were used in our experiments and were checked by weighing the successive 0·1 ml. amounts delivered. Statistical analysis showed that there was no great error introduced by measuring consecutive 0·1 ml. amounts, and that the results obtained conformed to the expected Poisson distribution.

Counting of the colonies. Colonies developing within the agar can be counted in the lateral illumination box used for counts of Petri dishes. The lower groove should be slightly notched close to the glass in order to support the base of the tube; the opposite groove should be elongated to accommodate the upper part of the tube. In this way the tube is held in position and can be rotated slightly if required. A thin film of agar (a very small fraction of the total) is produced on the sides of the tube during mixing, but any colonies that develop on this are readily detected.

As with the plate count, the coefficient of variation (Table 4) rises rapidly as the mean count decreases, e.g. with a mean count of 72·08 the coefficient of variation was 11·3 % and with a mean count of 7·62 it was 33·8 %. This indicates that the number of colonies should be as large as possible without introducing any error due to overcrowding. It is suggested that the number of colonies counted in a tube should lie between 30 and 300. The maximum will depend on the type of colony that is being counted. Counts of 250–300 have been made quite readily and as many as 400 with small streptococcal colonies. The surface-seeded drop count recommended by Miles & Misra appears to have a more limited range, the maximum count quoted being 100. Fivefold instead of tenfold dilutions may be used if required to obtain a convenient count, but in all our work this was unnecessary.

Application of the method. The agar-strip method has now been used extensively in studying the growth of pure cultures of various bacteria and has

proved satisfactory. With *Bacillus subtilis* the spreading growths did not interfere with counting to a greater degree than they did in the usual plating method. The suitability of the method for the examination of mixed populations remains to be tested. Fungi that produce rapidly spreading growths and bacteria which form mucoid colonies that tend to flow, may give rise to greater difficulties in tubes than they do in Petri dishes.

REFERENCES

Crone, P. B. (1948). The counting of surface colonies of bacteria. *J. Hyg., Camb.*, **46**, 426.

Esmarch, E. (1886). Ueber eine Modification des Koch'schen Plattenverfahrens zur Isolirung und zum quantitativen Nachweis von Mikroorganismen. *Z. Hyg.* **1**, 293.

Miles, A. A. & Misra, S. S. (1938). The estimation of the bactericidal power of the blood. *J. Hyg., Camb.*, **38**, 732.

Snyder, T. L. (1947). The relative errors of bacteriological plate counting methods. *J. Bact.* **54**, 641.

(*Received* 9 *November* 1949)

The Cultivation of *Haemophilus pertussis* in Partially Defined Liquid Media

By J. UNGAR, A. M. JAMES, P. W. MUGGLETON, H. F. PEGLER and E. G. TOMICH

Research Division, Glaxo Laboratories Ltd., Greenford, Middlesex

SUMMARY: Recently isolated virulent strains of *Haemophilus pertussis* grow well in a partially defined medium, but are more exacting in their growth requirements and conditions of growth than avirulent strains. The optimal pH for growth is 7·6–7·8. Mechanical agitation by stirring, spinning or aeration with either nitrogen or air, inhibits growth. Gaseous conditions, however, are critical in that there is an optimal relationship of surface area to volume of medium. Mechanical agitation and aeration stimulate the growth of avirulent strains.

Virulent strains require starch for growth, and there is a close relationship between virulence, specific agglutinability and starch requirement. Avirulent strains with low specific agglutinability grow readily in the absence of starch.

When starch in a standard medium is replaced by amylose, there is an increase in the final amount of growth. Amylopectin, glycogen and dextrans are about one-third as effective as starch; other carbohydrates, gums and inorganic adsorbents support little growth. Charcoal can replace starch, but has only *c.* 70% of its effect.

The amino-acid requirements of *H. pertussis* are satisfied by acid-hydrolysate of casein, the optimal concentration being 7%. All strains utilize aspartic and glutamic acids, serine, threonine, glycine, alanine and proline, but the rate of utilization is greater with virulent than with avirulent strains.

For good growth yeast extract is needed but can be replaced by nicotinamide, nicotinic acid or cozymase, provided sulphur-containing amino-acid is also present. Virulent strains of *H. pertussis* require a sulphur-containing acid for growth, which may be supplied by yeast extract. Cysteine, cystine or glutathione, but not methionine, act as sources of essential sulphur.

For many years the optimal medium for the cultivation of *Haemophilus pertussis* has been the solid medium originally recommended by Bordet & Gengou (1906). Strains grown on this medium maintain their biological properties unaltered for varying lengths of time. As the preparation of this medium necessitates the addition of either human or animal blood, Hornibrook attempted to replace it by a fluid medium (Hornibrook, 1939). Cohen & Wheeler (1945) modified Hornibrook's medium by adding copper and ferrous sulphates and increased the amounts of potassium phosphate, magnesium chloride and soluble starch. We used Cohen & Wheeler's modification as a basal medium, having first confirmed that the organism grew well in it.

Our investigation was primarily intended to establish whether liquid partially defined culture media would permit growth of organisms suitable for vaccine production, that is to say, of bacilli that maintained the antigenic properties characteristic of a freshly isolated strain. It was considered probable that the physical and nutritional requirements of the organism would need careful control under the particular conditions of growth in the medium. The purpose of this communication is to report a detailed study of these requirements and of

the conditions necessary for growth of strains having high antigenic activity. This activity was established by testing the vaccines made from them on rabbits and mice. The results of these tests will be reported elsewhere.

EXPERIMENTAL

Cultures. The strains of *H. pertussis* were isolated from Bordet-Gengou cough plates and subcultured on fresh Bordet-Gengou slopes, which were incubated at 37° and showed good growth on the second day. Each strain was freeze-dried (McLeod, 1944) immediately after isolation to avoid changes in biological properties, which often occurred with repeated subculture on Bordet-Gengou medium. We have stored strains in this way for several years without loss of virulence or agglutinability.

To determine the virulence of the strains, washed saline suspensions were made to contain $40{,}000 \times 10^6$ organisms/ml. Suitable dilutions of this were made with saline and introduced in quantities of two drops into the nostrils of several anaesthetized mice (Burnet & Timmins, 1937). It was found that the virulent strains killed all the mice in 10 days, or less, after intranasal inoculation of 400×10^6 bacilli or fewer. Post-mortem examination of the mice showed consolidation of the lungs, from which the strain was recovered.

The agglutinability of a strain was determined by titrating serial dilutions of rabbit Phase I pertussis antiserum against equal volumes of a formalin-killed suspension of the organisms. The final serum dilution that agglutinated the suspension was used as a measure of the agglutinability of the strain.

Inoculum. The organisms were subcultured only twice on Bordet-Gengou slopes before testing. Two-day-old cultures were washed off the slope with sterile saline, centrifuged and resuspended in sterile saline to an opacity corresponding to about 2000×10^6 organisms/ml. Of this suspension, 0·2 ml. was added to each 100 ml. of partially defined medium.

Partially defined medium. The liquid partially defined medium contained the constituents suggested by Cohen & Wheeler (1945) dissolved in distilled water, and the pH was adjusted to 7·6. The medium was brought to the boil and a mucilage of soluble starch was poured in with continuous stirring to give a final concentration of 0·15 %. The medium was filtered whilst hot and allowed to cool, readjusted to pH 7·6, dispensed into the required containers and autoclaved at 20 lb. pressure for 20 min. At this stage it was clear, pale yellow and free from deposit.

Estimation of amount of bacterial growth. When *H. pertussis* was incubated in this medium there was no visible growth for 2 or 3 days. Bacterial growth is commonly measured by direct count or turbidity, either by comparison of the suspension with a standard suspension or by use of a photoelectric absorptiometer. It was impossible to obtain a suitable counting chamber and the following method was therefore adopted. A known volume of a suitably diluted suspension of the organisms was smeared on a clean microscope slide and carefully dried by heat. The area of the smear was determined by projecting it on to a ground-glass screen and measuring the enlarged image with a planimeter, from

which the area of the smear itself could be calculated. The organisms were then fixed with methanol, stained with carbol fuchsin and counted with a $\frac{1}{12}$ in. oil-immersion objective and an eyepiece graticule. The average number of organisms in 100 squares at different parts of the field was determined, the graticule having been calibrated against normal human erythrocytes (assumed diameter 7μ.). The number of organisms/ml. was calculated from the formula:

$$\text{Number of organisms} = \text{Average per square} \times \frac{\text{Area of smear}}{\text{Area of square on graticule}} \times \frac{1}{\text{Vol. of suspension}} \times \text{Dilution.}$$

The method gave reproducible results with different concentrations of organisms. Table 1 shows the total count by the above method and the count estimated by opacity measurements with Brown's standard tubes (Burroughs Wellcome and Co.) of bacterial suspensions from a 14-day culture grown in the liquid semi-defined medium and from a 24 hr. culture on Bordet-Gengou medium. The ratio of the total count to the opacity count is approximately 1·8 for the liquid medium and 1·3 for Bordet-Gengou medium. This difference is probably due to different optical properties resulting from morphological differences in the constituent cells.

Table 1. *Relationship between opacity (by Brown's tubes) and total counts (by present method) in suspensions of* Haemophilus pertussis

Medium and growth conditions	Number of cells estimated by ($\times 10^6$/ml.) Opacity (a)	Total count (b)	b/a
Partially defined medium, 14-day culture	18,000	32,000	1·8
Organisms washed off and suspended in saline	3,500	6,700	1·9
	3,500	5,800	1·7
	1,600	2,800	1·75
	1,600	3,000	1·85
Bordet-Gengou medium, 24 hr. culture	10,800	11,000	1·0
Organisms washed off and suspended in saline	10,800	15,200	1·4
	10,000	12,600	1·2
	7,300	7,700	1·1
	7,300	8,400	1·2
	7,300	11,000	1·5
	3,800	4,100	1·1
	3,800	6,000	1·6

As a final check on the bacterial count the total nitrogen contents of suspensions of different virulent strains were estimated (Table 2). Total nitrogen/10^{12} organisms remained constant, thus confirming that the nitrogen content of the bacterial cell could be used as an index of the number of organisms present. The opacity method, being the most convenient, was adopted. In all the tables the growth is expressed as the total count (i.e. opacity × the appropriate factor) in millions of organisms/ml.

Table 2. *Relationship between opacity and total Kjeldahl-nitrogen in suspensions of seven different strains of* Haemophilus pertussis

Strain	Number of organisms estimated by opacity ($\times 10^6$/ml.)	Total N (g./ml.)	Total N (g./10^{12} cells)
1	137,000	0·0048	0·035
2	114,000	0·0046	0·040
3	114,000	0·0045	0·032
4	91,000	0·0042	0·046
5	57,000	0·0023	0·040
6	34,000	0·0012	0·035
7	28,000	0·0012	0·042

THE PHYSICAL CONDITIONS FOR THE GROWTH OF *HAEMOPHILUS PERTUSSIS*

Size of inoculum. The inoculum was varied from 4·5 to 350×10^6 organisms/ml. We found that even a 70-fold increase in size of the initial inoculum had little effect on the lag period or growth of the organisms (cf. Fisher, 1948).

Effect of pH. The partially defined medium was divided into several portions, which were adjusted to different pH values over the range 6·6–8·6. These samples of medium were then dispensed in 100 ml. amounts in 10 oz. medicine bottles and sterilized. There was no change in the pH on autoclaving. The media were inoculated with various virulent strains and incubated at 37° for 9 days. The strains grew over the range 6·6–8·6 and grew best at pH 7·60–7·85 (Fig. 1). In all succeeding experiments with the partially defined medium the initial pH was 7·6.

The gaseous requirements for growth in liquid partially defined medium

Nitrogen. The partially defined medium was dispensed in 800 ml. amounts in 2 l. conical flasks, which were autoclaved and incubated. Oxygen-free nitrogen was bubbled through concentrated sulphuric acid and then distilled water, passed through sterile air-filters, and slowly bubbled through the medium in two flasks and above the medium in a third flask; a fourth flask, without passage of gas, served as control. The media were inoculated with virulent organisms and incubated for 5 days; there was no growth in the media through which the nitrogen was bubbled or over which the nitrogen was passed. The count in the control medium was 3600×10^6 organisms/ml. It appeared that the nitrogen was giving rise to unfavourable anaerobic conditions.

Air. The above experiment was repeated, the nitrogen being replaced by washed and filtered air, using a virulent and an avirulent strain. The growth of the avirulent strain was greater in the aerated medium than in the unaerated medium (Table 3). The virulent strain failed to grow in medium through which the air was passed. After 4 days' incubation a sample seeded on Bordet-Gengou medium proved to be sterile, indicating that even the original inoculum was dead. This result was regularly experienced, even with marked variations in the rate of aeration. The unaerated control medium showed good

Table 3. *Growth of an avirulent strain of* Haemophilus pertussis *with different conditions of aeration*

Conditions of growth	Growth after (days) (cells × 10^6/ml.) 1	5	11	13
Non-aerated medium	700	2,000	3,600	3,600
Air passed over medium surface	400	1,300	3,600	3,600
Air passed through medium	700	3,600	10,000	10,800

growth on the fourth day. It was clear that aeration increased the growth of an avirulent but inhibited that of a virulent strain.

Carbon dioxide. The organisms were grown in Winchester quart bottles, the space above the medium being filled with different mixtures of air and carbon dioxide. Growth occurred over the range of carbon dioxide concentration 0·03–25 % and was optimal between 0·03 and 10 % carbon dioxide. With more than 10 % carbon dioxide the growth after 12 days' incubation decreased rapidly as the carbon dioxide concentration increased. The pH of the medium also fell, which probably accounts for the inhibition of growth.

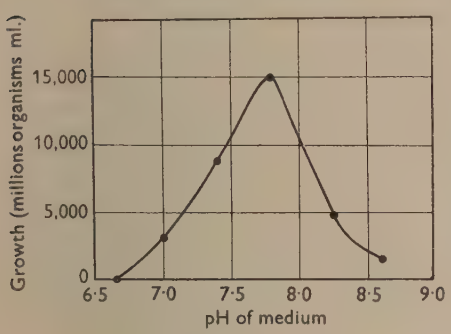


Fig. 1

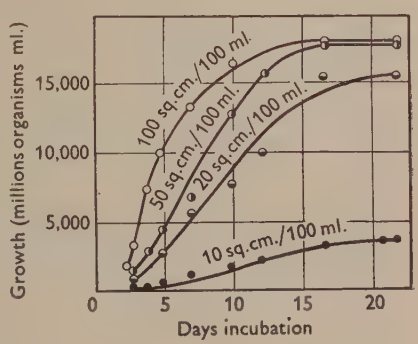


Fig. 2

Fig. 1. Variation of the growth of *H. pertussis* with the initial pH of the medium.

Fig. 2. Growth of *H. pertussis* in 10 oz. medical bottles containing different amounts of medium.

Mechanical agitation. Farrell & Taylor (1945) stated that mechanical agitation of the medium during incubation considerably decreased the period of incubation. We tried two methods of agitation: stirring the culture by means of an electric motor and rotating the culture container at an angle of forty degrees from the vertical. The method of mechanical agitation was immaterial; it always increased the growth of avirulent strains of *H. pertussis* and inhibited that of virulent strains.

Varying the surface/volume ratio. Cohen & Wheeler (1945) showed that the best growth was obtained in liquid medium dispensed into vessels so that there

was a very shallow layer of medium having a relatively large surface area, i.e. when there was a high surface/volume ratio. We confirmed this with vessels of various shapes and sizes. The medium was dispensed and sterilized in Winchester quart bottles, mould-culture flasks and 10 oz. flat medicine bottles to give surface/volume ratios ranging from 5 to 400 sq.cm./100 ml. of medium, i.e.

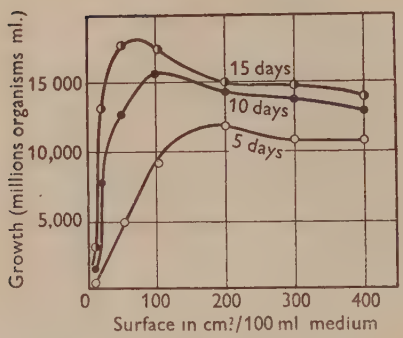


Fig. 3

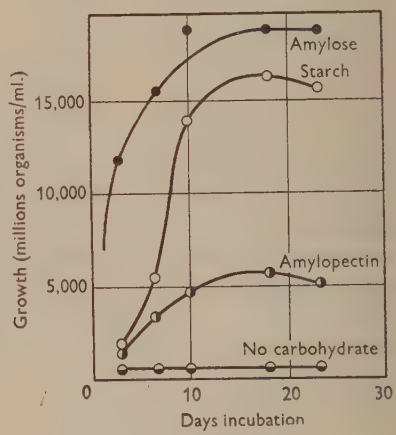


Fig. 4

Fig. 3. Variation of growth with the surface area after different times of incubation.

Fig. 4. Growth of *H. pertussis* in media containing different starch fractions.

ratios from 0·05 to 4. These were inoculated with a virulent strain and incubated at 37°. Fig. 2 shows the mean growths for different surface/volume ratios plotted against the period of incubation. As the surface/volume ratio increased, so did the initial growth. The maximum growth obtained with the larger surface/volume ratios was the same, but the time required to reach this value decreased with increasing values of this ratio. Fig. 3 shows growth after 5, 10 and 15 days' incubation plotted against different surface/volume ratios. The optimal surface/volume ratio decreased as the period of incubation increased. Surface/volume ratios in excess of the optimal, for any given period of incubation, appeared to be slightly inhibitory. This is consistent with the inhibitory effect of aeration. In another experiment a virulent strain was grown in 10 oz. medicine bottles containing varying amounts of medium; shaking the bottles for 5 min. each day inhibited the growth only when the surface/volume ratio was not optimal. Shaking bottles containing the optimal amount of medium had no effect on the growth. Growth under optimal conditions was diffuse, whereas under other conditions the growth occurred as a pellicle on the surface of the medium.

THE NUTRITIONAL REQUIREMENTS OF *HAEMOPHILUS PERTUSSIS*

The main constituents of the medium were carbohydrate, amino-acids, yeast dialysate and certain inorganic salts.

Carbohydrate requirement

Previous workers have stressed that starch is essential for the growth of *H. pertussis*. Hornibrook (1939) deduced the utilization of starch by the organisms from the iodine colour reactions of the medium before and after growth. Pollock (1949) showed that, during the growth of a 'modified' strain, unsaturated fatty acids were produced and he suggested that they were adsorbed on the starch. Schoch & Williams (1944) showed that the adsorption of unsaturated fatty acids on starch caused the normally blue iodine-starch complex to assume a brownish red colour. It may well be that *H. pertussis* produces fatty acids and that the change in colour of the iodine-starch complex is not due to utilization at all, but to adsorption of fatty acids.

Starch. The type of starch appeared to be immaterial; after 16 days' incubation the counts in media containing soluble, maize or potato starch were respectively 15,500, 13,500 and 16,000 $\times 10^6$ organisms/ml. We investigated the different starch requirements of twenty strains of *H. pertussis* and two strains of *H. parapertussis*. Media were made up containing the usual amount of starch (0·15 %) or without starch; three lots of each medium were inoculated. Virulent strains of *H. pertussis* grew only in the presence of starch, whereas avirulent strains and the two strains of *H. parapertussis* grew as readily without starch (Table 4). Agglutinability by rabbit Phase I pertussis antiserum was high for the virulent strains, low for organisms growing readily in the absence of starch and nil for avirulent strains. There thus seems to be a correlation between virulence, agglutinability and starch requirements, in that a virulent, agglutinable strain of *H. pertussis* will grow only in the presence of starch, while an avirulent, poorly agglutinable strain grows readily in its absence. *H. parapertussis* formed a diffuse turbidity in the culture medium and coloured it dark-brown, whereas *H. pertussis* grew with a pellicle on the surface and did not darken the medium.

Concentration of starch. Table 5 shows the effect of simultaneously varying the concentrations of starch and casein hydrolysate in the partially defined medium. Other virulent strains of *H. pertussis* behaved similarly. From these results we conclude that starch is essential for the growth of virulent strains but that concentrations of starch above 0·15 % do not improve growth. In this we differ considerably from Fisher (1948), who stated that the optimum concentration was 1·5 to 2·0 %.

Replacement of starch. Hornibrook (1939) stated that it was possible to replace the starch in the medium by another polysaccharide, β-dextrin, but that substances such as glycogen, saliva-hydrolysed starch, glucose, lactose, maltose, laevoglucosan, gum-acacia, mucin and agar could not do so. We found that starch could be replaced only by one of its constituents, namely, amylose; substances such as dextrin, glycogen, Cori ester, dextran, salicin and amylo-

Table 4. *Growth of different strains of* Haemophilus pertussis *in media with or without starch*

Strain	Virulence after 0, 4 and 12 days growth	Agglutination titre: 12 days in liquid media	Agglutination titre: 2 days on Bordet-Gengou	Growth (cells × 10^6/ml.) after (days): 4, Starch	4, No starch	12, Starch	12, No starch
82E	+ + +	1/10,000	1/16,000	1,200	0	20,000	2,000
83E	+ + +	1/10,000	1/16,000	2,200	0	17,000	0
102E	+ + +	1/10,000	1/2,000	3,500	0	18,000	0
104E	+	1/10,000	1/16,000	1,500	1,600	17,500	14,500
105E	—	1/500	1/1,600	1,500	1,200	18,000	18,000
146E	—	1/500	1/1,000	1,200	1,000	7,200	6,500
154E	—	1/500	1/1,000	1,200	2,000	7,200	14,000
167E	—	1/500	1/1,000	1,200	2,000	7,200	11,000
169E	*H. parapertussis*	1/2,500	1/500	3,500	3,600	20,000	20,000
191E	+ + +	1/10,000	1/20,000	1,200	0	18,000	0
192E	+ + +	1/10,000	1/16,000	2,000	0	20,000	0
193E	+ + +	1/10,000	1/16,000	700	0	17,000	0
196E	+ + +	1/5,000	1/16,000	1,000	0	17,000	0
197E	+ + +	1/10,000	1/16,000	1,200	0	11,000	0
199E	+ + +	1/10,000	1/8,000	1,000	0	17,000	0
203E	+	1/10,000	1/4,000	1,200	100	18,000	7,200
204E	+ + +	1/5,000	1/8,000	1,200	0	10,000	0
207E	*H. parapertussis*	1/5,000	1/250	3,500	4,200	20,000	19,000
209E	+ + +	1/10,000	1/16,000	1,500	0	18,000	0
210E	+ + +	1/10,000	1/16,000	1,000	0	14,500	1,800

+ = 50 %; and + + + = all mice dead in 10 days with 200 × 10^6 organisms.

Table 5. *The growth of a virulent strain of* Haemophilus pertussis *in media containing different concentrations of casein hydrolysate and starch*

Casein (%)	Starch concentration (%): 0·03	0·15	0·5	1·0
	Growth (cells × 10^6/ml.) on 14th day			
1	—	5,500	6,000	7,300
4	8,600	17,200	—	—
4	—	12,000	13,000	13,600
8	—	15,800	15,800	15,800
12	—	5,900	6,600	6,000
16	—	5,700	5,900	5,900

pectin allowed only limited growth. Amylose and amylopectin fractions prepared from potato starch (Haworth, Peat & Sagroth, 1946) were tested at a concentration of 0·15 % in place of the potato starch. In the initial stages of incubation, growth was more rapid in the medium containing the amylose (Fig. 4); there was, however, little difference between the final stationary populations in the amylose and the starch media. Amylopectin, on the other hand, permitted only limited growth.

The experiment was repeated with soluble starch and the corresponding amylose and amylopectin, and with a mixture of amylose and amylopectin in the same proportions as they occur in starch (Table 6). Again the best and

most rapid growth occurred in the medium containing amylose in place of the starch. There was no difference between the growth in the starch medium and the one containing a mixture of 1 part of amylose and 4 parts of amylopectin. The growth of the organisms in the different starch fractions did not affect agglutinability on the ninth day.

Table 6. *Growth of a virulent strain of* Haemophilus pertussis *in media containing different starch fractions*

	Growth (cells × 10^6/ml.) after (days)			
	4	9	16	22
Soluble starch	2,200	15,000	15,500	14,000
Soluble amylose	11,000	18,000	18,500	17,500
Soluble amylopectin	7,000	9,000	9,200	7,400
Mixture of amylose (1 part) and amylopectin (4 parts)	2,300	11,500	13,000	12,200

When starch was replaced by the following substances (0·15 %) the relative growths of a virulent strain at the 9th day, expressed as percentage of growth with starch, were: amylose (135), mannitol (46), glycogen (38), amylopectin (35), dextrin (32), Cori ester (24), salicin (23), dextran (23), sucrose (18), maltose (16), lactose (16), gum tragacanth, gum acacia or gum arabic (6), glucose (5) and apple pectin, agar or agar pectin (0).

Many organisms produce starch or starch-like substances from Cori ester (Hehre, Carlson & Neill, 1947), but neither a virulent nor an avirulent strain of *H. pertussis* formed starch or dextrin as shown by the iodine test.

Adsorbent effect of starch. In a study of the growth of a strain of *H. pertussis*, Pollock (1947) showed that the blood in the Bordet-Gengou medium could be replaced by charcoal and that charcoal could replace the starch in a semi-defined medium. There thus appeared to be two possible functions of the starch required for the growth of a virulent strain: it might act either as an adsorbent for toxic substances or as a source of carbohydrate.

These hypotheses were tested with virulent and avirulent strains. If the starch is acting primarily as an adsorbent then it should be possible to replace it by other absorbents. It was found that a virulent strain grew in a starch-free medium provided that charcoal was present.

The medium without starch, at pH 7·6, was dispensed in 250 ml. amounts in 1 l. conical flasks and in 100 ml. amounts in 10 oz. flat medicine bottles. Charcoal (acid-washed Sutcliffe Speakman No. 5) was added to give 0·1 and 0·4 % (w/v) respectively. In addition, an equivalent amount of charcoal was suspended in saline, tied up in a cellophan bag (grade 300, McMillan and Marshall) and put in the medium. As a control a starch solution was enclosed in a cellophan bag and placed in another flask of medium. The medium containing charcoal supported growth, but the final stationary population was less than that in the medium containing starch. Growth in the medium in which the charcoal was enclosed in a cellophan bag was almost equal to that in the complete medium. Organisms grown in the presence of starch were highly agglutin-

able and showed no change in morphology. From unpublished experiments, however, it appears that starch may not act solely as an adsorbent of toxic products. The following substances (at 0·1 %, w/v) could not replace starch: egg-albumin, alumina, silica gel, gelatin, agar.

Amino-acid requirements

Casein hydrolysate. The amino-acids required for growth are supplied by a casein hydrolysate, which was prepared by refluxing technical grade lactic casein with 6N-HCl for 18 hr. and then removing as much HCl as possible by vacuum distillation. The hydrolysate was then diluted with water and partially decolorized by boiling with charcoal, filtered through kieselguhr and adjusted to pH 6·7 with caustic soda; the insoluble tyrosine was removed by filtration, and 40 ml. of this hydrolysate were included in each litre of the medium, giving a total amino nitrogen concentration of about 1 mg./ml.

The casein hydrolysate usually used in the preparation of the semi-defined medium contained 16 % sodium chloride. It was necessary to decrease this concentration before high casein contents could be tested, because a high salt content is inhibitory to the growth of most micro-organisms. The casein hydrolysate was passed through an ion-exchange resin, which decreased the salt content to 5·9 %. Various concentrations of effluent were added to the medium, the final NaCl content being adjusted to 0·89 %. Growth on the 14th day was greatest with a casein concentration of about 7 %; with higher concentrations growth was inhibited.

'*Vitamin-free' casein hydrolysate.* The standard casein hydrolysate was compared in growth-promoting activity with 'vitamin-free' casein hydrolysed by hydrochloric acid, sulphuric acid or barium hydroxide. Media containing either acid- or alkaline-hydrolysed casein differed little. The apparent difference between the 'technical' and the 'vitamin-free' casein was further studied (Table 7). Solid 'technical' casein was refluxed for 12 hr. with ethanol to remove residual fat and traces of certain vitamins. The casein was filtered off, dried and hydrolysed. Media were made up containing this 'vitamin-free' casein hydrolysate or the original technical casein hydrolysate. Initial growth was slightly greater in the latter but the final bacterial count was the same in both media. Further, the ethanolic extract of the casein was slightly inhibitory, owing perhaps to the fatty substances it contained.

Table 7. *Effect of different casein hydrolysates on growth of a virulent strain of* Haemophilus pertussis

(Size of inoculum: 400×10^6 cells/100 ml. of medium.)

Casein	Method of hydrolysis	Charcoal treatment	Growth (cells $\times 10^6$/ml.) after (days)	
			7	10
Technical	6N-HCl	Yes	11,000	18,500
Vitamin-free	$Ba(OH)_2$	No	3,600	10,000
Vitamin-free	6N-HCl	Yes	6,300	10,000
Vitamin-free	6N-HCl	No	5,400	12,500
Technical	H_2SO_4	No	4,500	10,500

Purification of the casein hydrolysate. Casein acid-hydrolysate was purified by various methods (Table 8). Boiling the casein hydrolysate with acid-washed charcoal in alkaline solution removed some of the growth-promoting substances; similar charcoal treatment in acid solution removed less. Hydrolysates filtered through kieselguhr or paper pulp gave as good growth as did untreated ones. This method of treatment, however, gave a very dark brown medium. Boiling the complete medium with acid-treated charcoal, readjusting the pH to 7·6 and then filtering gave a medium that supported little, if any, growth.

Table 8. *Effect of charcoal* (C) *treatment of casein hydrolysate on growth of virulent strain of* Haemophilus pertussis

Treatment	pH of medium	Growth (cells × 10^6/ml.) after (days)				
		2	5	9	12	21
Filtered through kieselguhr	7·52	0	250	6,300	11,000	11,500
Filtered through paper pulp	7·57	300	6,300	6,300	11,500	11,500
Boiled once with C *at* pH 4, *filtered*	*7·53*	*350*	*4,500*	*7,200*	*11,000*	*11,000*
Boiled twice with C at pH 4, filtered	7·50	350	6,300	7,400	13,000	15,500
Boiled once with C at pH 4, neutralized and boiled with C at pH 7	7·60	200	270	1,800	3,600	3,200
Neutralized and boiled once with C at pH 7	7·60	200	1,800	5,400	10,000	6,500
Neutralized boiled twice with C at pH 7	7·48	200	4,200	3,000	6,800	5,500

The results in italics are for media containing untreated casein hydrolysate.

Utilization of amino-acids during growth. Three strains of *H. pertussis* were grown in the standard medium, the growth was measured after different periods of incubation. At the end of 15 days' incubation the cells were removed from the medium by centrifuging, the supernatant was preserved under toluene, and its amino-acids were assayed by paper chromatography (Dent, 1948; Consden, Gordon & Martin, 1944). Pl. 1 shows the developed chromatograms obtained with 0·022 ml. of supernatant fluids from cultures of a virulent (83E) and an avirulent strain (154E) on Whatman No. 4 filter-paper. Aspartic acid, glutamic acid, serine, threonine, glycine and alanine were utilized to a greater or lesser extent, depending on the period of incubation. Proline (which cannot be seen in the photograph, because the proline-ninhydrin complex is yellow) was completely used after 15 days. The size and intensity of the spots from the culture fluids were compared visually with those of the same spots from the uninoculated control solution, to give a rough measure of the amounts of amino-acids utilized (Table 9). Table 9 also records the approximate amino-acid contents of the culture fluids estimated by the methods of Pope & Stevens (1937). Fig. 5 shows the growth and the corresponding curve of total amino-acid utilization for a virulent strain. This amino-acid utilization suggested that it might be advantageous to fortify the medium with amino-acids or with more casein hydrolysate, but the addition of aspartic acid, glutamic acid, serine and glycine neither shortened the lag nor increased the stationary population.

Table 9. *Residual amino-acids present in the culture filtrates as percentage of the initial concentration*

Days incubation	Total amino-nitrogen (mg./100 ml.)	Aspartic acid	Glutamic acid	Serine	Glycine	Threonine	Alanine	Proline	Growth (cells × 10^6/ml.)
Virulent strain:									
0	90	100	100	100	100	100	100	100	0
5		40	20	10	20	100	80	100	9,200
7		0	0	0	0	70	50	—	12,000
9		10	0	0	0	70	50	—	18,200
12		0	0	0	0	70	50	0	15,000
15	32	0	0	0	0	50	50	0	20,000
Avirulent strain:									
0	90	100	100	100	100	100	100	100	0
5		100	100	100	100	100	100	100	2,000
7		80	80	50	80	100	100	—	8,100
9		30	20	10	50	100	100	—	10,600
12		0	0	0	0	50	50	—	15,200
15	21	0	0	0	0	50	50	0	19,200

* The figures for amino-acid concentration are based on the relative sizes and intensities of spots on the paper chromatogram.

Strain	Total percentage drop in amino-nitrogen in 15 days: By Pope & Stevens method	By chromatography
Virulent	64	50
Avirulent	78	50

Other nutritional requirements of Haemophilus pertussis

Yeast dialysate. Yeast extract increases growth in liquid partially defined medium (Hornibrook, 1939). We found that a yeast infusion had the same growth-promoting properties as a yeast dialysate from the same amount. It thus appears that during dialysis in distilled water at 80° for 18 hr. the necessary growth factors pass into the dialysate, and are not heat-labile. All media used contained yeast dialysate, which was stored under toluene at 5° until required for use. A virulent strain of *H. pertussis* did not grow in medium without yeast dialysate, but grew luxuriantly when a small amount was added. A twenty-fold increase in concentration did not improve growth further.

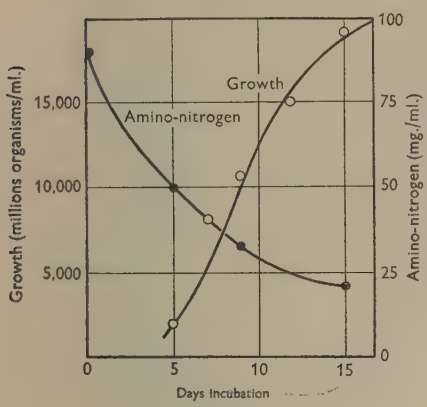


Fig. 5

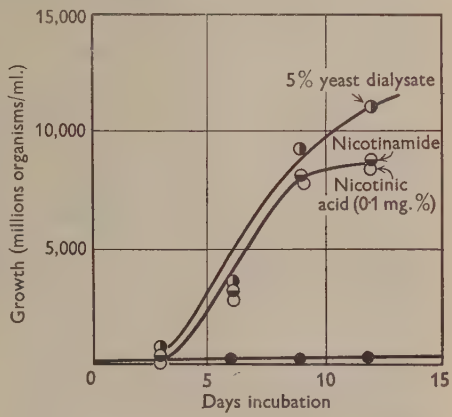


Fig. 6

Fig. 5. Growth of *H. pertussis* and the utilization of amino-nitrogen in the medium.

Fig. 6. Growth of *H. pertussis* in media in which the yeast dialysate is replaced by nicotinamide and nicotinic acid.

Replacement of yeast dialysate. Hornibrook (1939) reported that yeast dialysate in the partially defined medium could be replaced by nicotinic acid or nicotinamide. *H. pertussis* grew readily in the presence of 0·1 mg. nicotinic acid or nicotinamide/100 ml., in place of yeast dialysate, but the final bacterial population was decreased by about 20 % (Fig. 6). A preparation of cozymase (Sumner, Krishman & Sisher, 1947) at 0·06 mg./100 ml., supported the growth better than did nicotinamide. Weight for weight the preparation of cozymase was more active than the nicotinamide or nicotinic acid, suggesting that the organisms synthesize cozymase from nicotinic acid.

Organic sulphur. Hornibrook (1939) stated that organic sulphur is essential for the growth of the organism. Yeast dialysate contains organic sulphur in methionine and cystine (Diemain & Fresenius, 1947) and must be omitted from the medium for a study of the sulphur requirements of *H. pertussis.* In the medium used yeast dialysate was replaced by nicotinamide, and cystine, cysteine, methionine or glutathione added as required. We confirmed that a

virulent strain required a sulphur-containing amino-acid for growth (Table 10), usually supplied in yeast dialysate and by cysteine. When yeast dialysate is replaced by nicotinamide, cysteine, cystine or glutathione must be added for growth to occur. Methionine cannot supply the organic sulphur required.

Table 10. *Growth of* Haemophilus pertussis *in media containing different sulphur-containing amino-acids*

Yeast or nicotinamide added	Source of sulphur	Growth (cells × 10^6/ml.) after (days)			
		3	7	11	14
Yeast	Cysteine	1,800	12,000	13,000	15,000
Nil	Nil	2,000	2,000	2,000	2,000
Yeast	Nil	1,800	12,000	13,000	15,000
Nicotinamide	Cystine	1,400	7,200	13,000	13,000
Nicotinamide	Cysteine	2,000	5,200	12,000	13,000
Nicotinamide	Methionine	2,000	3,200	4,100	7,000
Nicotinamide	Glutathione	2,000	5,200	11,000	14,500
Nicotinamide	Nil	2,000	2,000	2,000	2,000

CONCLUSION

The difference between virulent and avirulent strains of the same bacterial species has a practical as well as a theoretical interest, especially for those concerned with the preparation of prophylactic and therapeutic vaccines and antisera. Understanding of the fundamental biochemical differences that ultimately determine the intra-species differences in virulence is likely to be forwarded by a study of all differences in physical, chemical and biological properties that can be demonstrated between the two extreme types of strain.

In earlier papers (Ungar & Muggleton, 1948, 1949) we recorded different 'solubilities' of virulent and avirulent strains of *H. pertussis* in caustic soda and bile salt solutions, which completely 'dissolve' the virulent but not the avirulent strains. There was a similar contrast between the strains on treatment with aluminium phosphate precipitated *in situ*; there was simultaneous precipitation of virulent, but not of avirulent, strains.

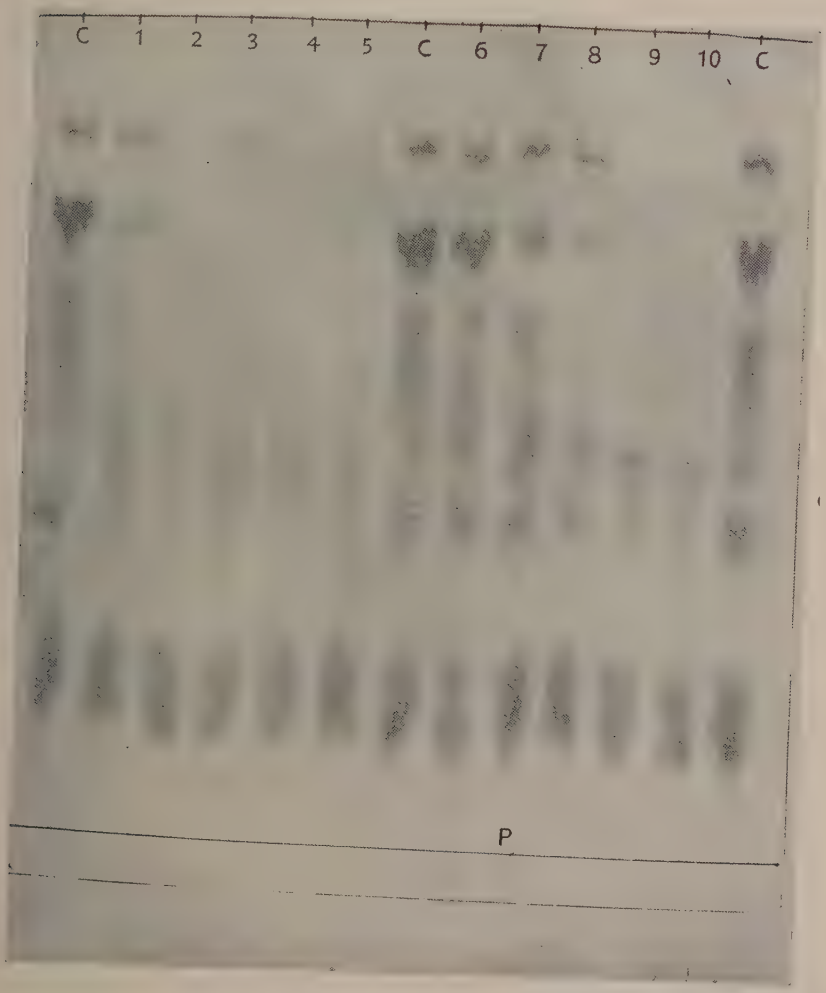
We have now demonstrated clearly two main nutritional differences in their relative needs for starch and amino-acids; there are also clear-cut differences between the effects of other environmental conditions such as supply of oxygen and pH. How far differences of this kind are related, and in what way, to the differences in physical properties recorded in our earlier papers and to differences in virulence, it is not yet possible to say, but it seems likely that they all depend on some fundamental biochemical divergence.

REFERENCES

Bordet, J. & Gengou, O. (1906). Le microbe de la coqueluche. *Ann. Inst. Pasteur*, **20**, 731.

Burnet, F. M. & Timmins, C. (1937). Experimental infection with *Haemophilus pertussis* in the mouse by intranasal infection. *Brit. J. exp. Path.* **18**, 83.

Cohen, S. & Wheeler, M. (1945). Pertussis vaccine prepared with Phase I cultures in fluid medium. *Report of the Division of Laboratories and Research N.Y. State Department of Health*, p. 39.



UNGAR, A. M. JAMES, P. W. MUGGLETON, H. F. PEGLER & E. G. TOMICH—THE CULTIVATION OF *H. PERTUSSIS* IN PARTIALLY DEFINED LIQUID MEDIA. PLATE 1

Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). Partition chromatography with paper. *Biochem. J.* **38**, 224.

Dent, G. E. (1948). Partition chromatography with paper. *Biochem. J.* **43**, 169.

Diemain, W. & Fresenius, W. (1947). Chemical composition of yeasts. *Dtsch. Lebensm. Rundsch.* **43**, 117.

Farrell, L. & Taylor, E. M. (1945). Notes on the production of Phase I pertussis vaccine in fluid medium. *Canad. J. publ. Hlth,* **36**, 326.

Fisher, S. (1948). The behaviour of *H. pertussis* in casein hydrolysate broth. *Aust. J. exp. Biol. med. Sci.* **24**, 299.

Haworth, W. N., Peat, S. & Sagroth, P. E. (1946). Amylose and amylopectin preparation. *Nature, Lond.*, **157**, 19.

Hehre, E. J., Carlson, A. S. & Neill, J. M. (1947). Production of starch-like material from glucose-1-phosphate by diphtheria bacilli. *Science*, **106**, 523.

Hornibrook, J. W. (1939). Cultivation of Phase I *H. pertussis* in a semi-synthetic liquid medium. *Publ. Hlth Rep., Wash.*, **54**, (2), 847.

McLeod, N. (1944). A simple and rapid method for the preservation of cultures by drying under vacuum. *Inst. Med. Lab. Techn.* p. 92.

Pollock, M. R. (1947). Growth of *H. pertussis* on medium without blood. *Brit. J. exp. Path.* **28**, 295.

Pollock, M. R. (1949). The effects of long-chain fatty acids on the growth of *Haemophilus pertussis* and other organisms. *Symp. Soc. exp. Biol.* **3**, 193.

Pope, C. G. & Stevens, M. F. (1937). The determination of amino-nitrogen using a copper method. *Biochem. J.* **33**, 1070.

Schoch, T. J. & Williams, C. B. (1944). Adsorption of fatty acid by the linear component of corn starch. *J. Amer. chem. Soc.* **66**, 1232.

Sumner, J. B., Krishman, P. S. & Sisher, Eleanor B. (1947). An improved method for the preparation of co-enzyme I. *Arch. Biochem.* **12**, 19.

Ungar, J. & Muggleton, P. W. (1948). Aluminium phosphate as precipitant of agglutinable and non-agglutinable strains of *H. pertussis*. *Nature, Lond.*, **162**, 734.

Ungar, J. & Muggleton, P. W. (1949). The relationship of the aluminium phosphate precipitation of organisms of *Haemophilus pertussis* strains to their other biological properties. *J. gen. Microbiol.* **3**, 353.

EXPLANATION OF PLATE

Chromatogram showing the utilization of amino-acids during the growth of a virulent and an avirulent strain of *H. pertussis*. 1–5: culture fluids after 5, 7, 9, 12 and 15 days' growth of the virulent strain (83E); 6–7: culture fluids after 5, 7, 9, 12 and 15 days' growth of the avirulent strain (154E); *C*: control, unseeded culture medium; *P*: phenol-water front.

(*Received* 10 *November* 1949)

Enzymes of *Clostridium welchii* Type A and *Clostridium histolyticum* that Disintegrate Decalcified Human Tooth Dentine

By D. G. EVANS and A. S. PROPHET*

Department of Bacteriology and Turner Dental School, University of Manchester

SUMMARY: Enzymes elaborated by *Clostridium histolyticum* and by *Cl. welchii* type A that disintegrated the organic component of human tooth dentine were compared with those attacking muscle collagen.

The potency of specific antisera to neutralize the enzymes disintegrating the organic component of dentine was determined by using as indicator finely divided decalcified dentine suspended in an agar gel.

By comparing the potency values of the sera with those obtained when muscle collagen preparations were used as indicators, it was evident that the organic component of dentine was attacked by the collagenases of *Cl. welchii* type A (κ antigen) and *Cl. histolyticum* (β antigen) and probably by the γ antigen of *Cl. histolyticum*. The λ antigen of *Cl. welchii* type B had no action on decalcified dentine.

Oakley, Warrack & van Heyningen (1946) showed that the collagenase (κ antigen) of *Clostridium welchii* type A, which attacks muscle collagen, was antigenically distinct from the other known antigens produced by this organism and devised methods to determine the anticollagenase potency of specific antisera, using collagen preparations as indicators. Evans (1947) investigated the production, by certain species of *Clostridium*, of enzymes disintegrating finely divided hide-powder, which, although a convenient and rich source of collagen, was not regarded as a pure substrate; and its disintegration not considered a specific indication of collagenase. Of the organisms examined by this method, *Cl. histolyticum* and *Cl. welchii* type A were found to be the most active producers of enzyme. It was later reported by Oakley, Warrack & Warren (1948) that hide-powder was not a reliable indicator for collagenase, for this substrate was also attacked by a second enzyme, the *Cl. welchii* λ antigen which had no action on collagen. They suggested that during the preparation of hide-powder, the collagen was altered in some way so as to make it susceptible to attack by the λ antigen. They showed that λ antigen was produced by *Cl. welchii* types B, E and D, whereas collagenase was produced by types A, C, D and E. Oakley & Warrack (1950) also demonstrated that *Cl. histolyticum* produced two enzymes, each of which was antigenic, one a collagenase (β antigen) which attacked collagen and hide-powder, and the other (γ antigen) which attacked hide-powder but not collagen. Each of these enzymes was antigenically distinct from the lethal toxin of *Cl. histolyticum*.

Recently we showed (Evans & Prophet, 1950) that certain species of *Clostridium* and *Bacillus* formed enzymes which disintegrated the collagen-like matrix of human tooth dentine. Among the *Clostridium* species, *Cl. histo-*

* Nuffield Research Fellow.

lyticum and *Cl. welchii* type A were the most active, and the question was considered as to whether these enzymes were related to those attacking the collagen of muscle. The problem was most conveniently investigated by determining the potency of specific antisera to neutralize the enzymes responsible for disintegration of dentine collagen and comparing the results with those obtained when muscle collagen was used as indicator.

EXPERIMENTAL AND RESULTS

Preparation of dentine substrate

The dentine substrate was prepared by the method of Evans & Prophet (1950). Human tooth dentine was ground in a low-temperature ball-mill and the finely divided powdered dentine decalcified by 0·2N-HCl. The decalcified dentine powder, when washed free from acid and dried, served as the substrate. For testing the activity of enzymes, the powder was suspended in melted 4 % agar of neutral pH and pour-plates made in Petri dishes in which the particles of decalcified dentine were densely and uniformly distributed in a concentration of 0·1 g./100 ml. of agar.

Titration of enzymes

Dried ammonium sulphate precipitates from filtrates of broth cultures of *Cl. welchii* type A and *Cl. histolyticum* were titrated by a cup-plate method. Cups of 6 mm. diameter were cut with a cork-borer in decalcified dentine agar plates and approximately 0·1 ml. of increasing dilutions of a saline solution of the toxic precipitate were pipetted into each cup. The plates were incubated at 37° and after 24 hr. active dilutions produced around the cup a clear concentric zone in which the dentine particles were completely dissolved; the zone increased in size on incubating a further 24 hr., when the final readings were made. The average minimal effective dose (m.e.d.) of enzyme preparation chosen for the tests, was 0·008 mg./ml. for *Cl. histolyticum* and 0·15 mg./ml. for *Cl. welchii* type A. With each enzyme preparation one minimal effective dose (1 m.e.d.) gave a zone of 10 mm. diameter, while dilutions containing 10 m.e.d. or more gave 30 mm. zones.

Neutralization of enzymes by antisera

The cup-plate method was used to titrate the neutralizing potency of specific antisera against enzyme present in the enzyme preparations. The antisera had been prepared in horses and tested by Dr C. L. Oakley for neutralization of enzymes attacking collagen. Each antiserum was titrated by making saline dilutions and to each dilution an equal volume of a solution of the homologous enzyme preparation was added. Each serum dilution in the series differed from the next by 10 % or less. After standing at room temperature for 30 min., 0·1 ml. of each mixture was pipetted into the cups and the plates incubated at 37° for 48 hr. With most of the titrations each cup contained 100 m.e.d. of enzyme. With low titre sera, however, weaker concentrations of enzyme were used to obtain an end-point in the titration.

Table 1 gives the results of the tests with eight *Cl. welchii* antisera using

Table 1. *Neutralization by* Clostridium welchii *antisera of enzymes disintegrating* (*a*) *decalcified dentine and* (*b*) *collagen*

Antiserum	Neutralizing values using as indicator: Decalcified dentine	Collagen (anti-κ)
EX 1308 (Standard)	470	470
RX 5218	5600	4800
RX 5167	8700	7100
R 8480	85	80
RX 5457/36	5	5
EX 1055	2900	2400
R 5434	60	60
R 6423	<1	*c.* 1

serum EX 1308 as a standard, in terms of which the relative anti-enzymic activity of the other sera is expressed. The table also includes, for comparison, the anticollagenase values. It was evident that the neutralizing value of each *Cl. welchii* antiserum when tested with decalcified dentine as indicator, was of the same order as the anticollagenase value. This relationship suggests that the active antibody in both tests was in fact an anticollagenase, and that collagenase was responsible for the disintegration of decalcified dentine. It does not, however, follow from these findings that the organic component of dentine was identical with the collagen of muscle.

Table 2. *Neutralization by* Clostridium histolyticum *antisera of enzymes disintegrating* (*a*) *decalcified dentine,* (*b*) *collagen and* (*c*) *hide-powder*

Antisera	Neutralizing values using as indicator: Decalcified dentine	Collagen	Hide-powder
RR 2001 (Standard)	100	100	100
RR 2008	400	370	350
H 1982A	250	260	230
H 1982B	165	180	175
RR 2005	185	330	165
RR 2035	165	230	190

Table 2 gives the results of the titrations of six *Cl. histolyticum* antisera and the corresponding anticollagenase values. In this case serum RR 2001 was used as standard. There was not the same close relationship between the two sets of values in these tests as there was with the *Cl. welchii* antisera. The values for four sera agree very well and were presumably determined against collagenase in both cases. The values for each of the sera RR 2005 and RR 2035 however did not agree, suggesting that *Cl. histolyticum* produces, in addition to collagenase, another enzyme capable of attacking decalcified dentine. Oakley & Warrack (1950) in testing the same six *Cl. histolyticum* antisera with dyed hide-powder (azocoll) as indicator found that these two sera had potencies with that indicator which were different from their anticollagenase potencies. A typical set of their results with hide-powder is given in Table 2 for comparison. They

contended that these discrepant results were due to a second enzyme, which they named γ antigen, which attacked hide-powder but not collagen. It is highly probable that the discrepant results in titrating sera RR 2005 and RR 2035 with decalcified dentine as indicator were also due to this second enzyme γ.

Neutralization tests with dentine decalcified by mild treatment

The results with *Cl. histolyticum* antisera suggested that decalcified dentine was disintegrated not only by collagenase but also by an enzyme which attacked degraded collagen in the form of hide-powder. It was possible that the organic component of dentine was degraded during decalcification, making it susceptible to attack by enzymes which attacked degraded collagen. We could not investigate this problem by using as substrate the organic component as it occurred in its natural form, for it was not susceptible to enzyme attack until it was separated from the inorganic-organic complex by decalcification (Evans & Prophet, 1950). It was possible, however, to decalcify dentine by a mild acid treatment unlikely to cause degradation of the organic component and to use the preparation for titrating the *Cl. histolyticum* sera.

Table 3. *Neutralization by* Clostridium histolyticum *antisera of enzymes disintegrating* (*a*) *dentine decalcified at* pH 6·0 *and* (*b*) *collagen*

Antisera	Neutralizing values using as indicator: Dentine decalcified at pH 6·0	Neutralizing values using as indicator: Collagen (anti-β)
RR 2001 (Standard)	100	100
RR 2008	395	370
H 1982A	280	260
H 1982B	180	180
RR 2005	180	330
RR 2035	165	230

Pour-plates of finely divided dentine powder in 4 % agar were prepared, in which the dentine particles were uniformly distributed in a concentration of 0·4 g./100 ml. of agar. From these plates 6 mm. agar disks were cut and decalcified by placing in excess buffer solution (Evans & Prophet, 1950). Disks treated with McIlvaine phosphate-citric acid buffer at pH 6·0 (cf. Clark, 1928) were employed as indicator in the titration of the *Cl. histolyticum* sera. Serum dilutions were mixed with solutions containing 100 m.e.d./ml. of enzyme. Into each mixture a decalcified dentine agar disk was placed and the mixtures incubated at 37°. After 24 hr. disks from tubes where enzyme had not been entirely neutralized were completely clear, whereas those from tubes where neutralization had occurred contained visible particles of decalcified dentine. The results of these titrations are given in Table 3 together with the values obtained when collagen was used as indicator. Again the values for sera RR 2005 and RR 2035 did not agree with those for anticollagenase activity, suggesting that dentine decalcified by mild treatment is susceptible to attack by both collagenase of *Cl. histolyticum* and another enzyme, probably γ, and that

the susceptibility to the second enzyme is not due to the degradation of the organic component during decalcification.

Although decalcified dentine was apparently susceptible to attack by the γ antigen of *Cl. histolyticum*, it was not however attacked by the λ antigen of *Cl. welchii* type B. A preparation of λ antigen had no disintegrating action on decalcified dentine in a concentration 100 times the minimal dose active with hide-powder.

CONCLUSIONS

It was evident from these results that the organic component of human tooth dentine was attacked by the collagenases of *Cl. welchii* type A (κ antigen) and *Cl. histolyticum* (β antigen) and probably by the γ antigen of *Cl. histolyticum* but not by the λ antigen of *Cl. welchii* type B.

We should like to express our thanks to Dr C. L. Oakley for helpful advice and for supplies of antisera and λ antigen of *Cl. welchii* type B.

REFERENCES

Clark, W. M. (1928). *The determination of hydrogen ions*, 3rd ed. London: Baillière, Tindall and Cox.

Evans, D. G. (1947). The production by certain species of *Clostridium* of enzymes disintegrating hide-powder. *J. gen. Microbiol.* **1**, 378.

Evans, D. G. & Prophet, A. S. (1950). The disintegration of human dentine by bacterial enzymes. *Lancet*, i, 290.

Oakley, C. L. & Warrack, G. H. (1950). The Alpha, Beta and Gamma Antigens of *Clostridium histolyticum* (Weinberg & Séguin, 1916). *J. gen. Microbiol.* **4**, 365.

Oakley, C. L., Warrack, G. H. & Heyningen, W. E. van (1946). The collagenase (κ toxin) of *Cl. welchii* type A. *J. Path. Bact.* **58**, 229.

Oakley, C. L., Warrack, G. H. & Warren, M. E. (1948). The kappa and lambda antigens of *Cl. welchii*. *J. Path. Bact.* **60**, 495.

(*Received* 28 *November* 1949)

The Alpha, Beta and Gamma Antigens of *Clostridium histolyticum* (Weinberg & Séguin, 1916)

By C. L. OAKLEY and G. HARRIET WARRACK

The Wellcome Research Laboratories (Biological Division), Beckenham, Kent

SUMMARY: Three antigenic components (α, β, γ) are present in toxic culture-filtrates of *Clostridium histolyticum*. Of these α is the lethal and necrotizing toxin, β is a collagenase, and γ is a cysteine-activated proteinase which attacks altered collagen (e.g. hide-powder or azocoll) but not native collagen. The β and γ-enzymes both attack gelatin. By methods based on the properties of these antigens, *Cl. histolyticum* antisera can be tested for the corresponding antibodies.

Weinberg & Séguin (1916) described as *Bacillus histolyticus* an obligate anaerobe isolated by them from patients with gas gangrene. The organism tended to occur in pairs, was rather small (3–$5\mu \times 0{\cdot}5$–$0{\cdot}7\mu$), richly flagellate, vigorously motile in young cultures and in effusions, had large subterminal endospores and was actively proteolytic. It was pathogenic for guinea-pigs, mice and rabbits, less so for rats; intramuscular injection of culture led to extensive local destruction, splitting of the skin, extrusion of a haemorrhagic muscle pulp, denudation of the bone and sometimes to auto-amputation through a joint. Neither putrid changes nor gas formation occurred in the liquefying area, which frequently spread far beyond the injected limb.

There have been few subsequent records of the isolation of the organism, now called *Clostridium histolyticum* (see Peterson & Hall, 1922–3; Hall, 1923–4; Torrey, 1925). Other work has shown that it digests native and denatured proteins to amino-acids with production of ammonia, that it clots milk and later digests the clot (Blanc & Pozerski, 1920*a*) and haemolyses sheep red cells (Kahn, 1922). There is some question whether *Cl. histolyticum* is saccharolytic; Kahn (1922) found that it fermented maltose, lactose, xylose and inulin with production of acid; Hall (1922–3) found that all five strains examined by him failed to ferment glucose, fructose, maltose, lactose, sucrose, salicin, glycerol or inulin. These differences may perhaps be explained by Hoogerheide's work (1937) on the dissociation of *Cl. histolyticum* into saccharolytic non-pathogenic strains resembling *Cl. sporogenes*, and typical non-saccharolytic pathogenic *Cl. histolyticum*.

Descriptions of the microscopic changes produced by infection with *Cl. histolyticum* are given by Nasta (1922), who remarked on the extension of oedema far beyond the infecting organisms, and by Beckwith & MacKillop (1923–4).

Products detected in culture filtrates

The lethal and necrotizing toxins. Weinberg & Séguin (1916) observed that the culture-filtrates were toxic for guinea-pigs and rabbits; intravenous injection of sufficiently strong filtrates killed the animal in a few minutes; paralysis was followed by respiratory arrest. No author appears to have described a necrotic

lesion following intracutaneous injection of *Cl. histolyticum* filtrates, but Pasternack (1940) showed that the necrotic and degenerative changes produced in the internal organs by infection with the organism could be almost completely duplicated by intravenous injection of filtrates. Subcutaneous injection of filtrate led to boggy oedema and purplish discoloration of the skin in 3–4 days, followed by linear rupture of the skin and discharge of 'beet-soup fluid'; most animals then recovered, though some suffered exposure of bone or spontaneous amputation. Pozerski & Guélin (1938*b*) noted the production of an 'escarre' after subcutaneous injection of *Cl. histolyticum* filtrate into guinea-pigs. Gildemeister & Schlossberger (1932) showed that filtrates injected into the cornea led to swelling of the cornea and to formation of bubbles in it. No attempt seems to have been made to decide whether all these effects are due to the same substance.

Proteinases. Blanc & Pozerski (1920*a*) showed that filtrates of *Cl. histolyticum* digested proteins less readily than cultures did, and that, except in the case of gelatin, which was digested to amino-acids, the products of digestion were peptones. Filtrates of *Cl. sporogenes* were similarly proteolytic, but their activity was inhibited by normal serum and by ovalbumin, neither of which had any effect on the proteolytic effect of *Cl. histolyticum* filtrates (Blanc & Pozerski, 1920*b*; Pozerski & Guélin, 1938*a*). Bessey & King (1934) showed that *Cl. histolyticum* filtrates attacked gelatin much more vigorously than did filtrates of *Cl. sporogenes*; no pepsin or papain type enzymes or polypeptidases were present in filtrates of 72 hr. cultures. Weil & Kocholaty (1937) found that filtrates of 3-day cultures of *Cl. histolyticum* contained a proteinase, active against casein, clupein, Witte peptone and egg-albumin, that was activated by ferrous ions, by cysteine (better by both together) by manganese, nickel, cobalt and copper ions. All sulphydryl compounds used activated it (even the relatively insoluble thio-β-naphthol) but not hydrogen sulphide or cyanide. Oxygenation reversed the ferrous ion + cysteine effect; iodoacetic acid did not; high concentrations of hydrogen peroxide decreased the activity irreversibly. According to Kocholaty, Weil & Smith (1938) the proteinase appeared 6 hr. after inoculation of the medium, and was at its maximum after about 15 hr.; its concentration ran roughly parallel with the amount of bacterial growth, and there was no evidence that it was an intracellular enzyme. Kocholaty & Weil (1938) contended that by repeated subculture on gelatin or casein, *Cl. histolyticum* could be 'trained' to produce enzymes which attacked gelatin but not casein, and vice versa. This remarkable specificity of the proteinase could be abolished, either by growing the organism in gelatin plus the amino-acids necessary to make gelatin 'equivalent' to casein, or by activating the filtrates from either casein or gelatin cultures with ferrous ions and cysteine; the filtrates then attacked gelatin or casein indifferently (see also Kocholaty & Krejci, 1948). The proteinase was neutralized by *Cl. histolyticum* antisera.

On the other hand, Maschmann (1938) found that filtrates of *Cl. histolyticum* contained extracellular proteinases not activated by cysteine, and that only in the later stages of growth was a proteinase secreted that was activated by cysteine. van Heyningen (1940) claimed to have reconciled these discrepancies

by showing that colonies of *Cl. histolyticum* varied greatly in the proportions of two proteinases produced by them, and that it was possible to obtain strains which produced in early growth an extracellular proteinase activatable by cysteine and inhibited by iodoacetic acid, while older cultures (after 12 hr. incubation) contained an intracellular enzyme inhibited by cysteine.

A fibrinolysin has been described by Carlen (1939) and by Reed, Orr & Brown (1943).

Collagenases. It was noted by Weinberg & Séguin (1916) that while injection of cultures of *Cl. histolyticum* produced extensive destruction of muscle, filtrates attacked the connective tissue only (see also Hall & Peterson, 1922–3). This difference must, however, be relative, for Pasternack (1940) found that subcutaneous injection of filtrates led not only to extensive oedema with swelling and pseudo-colloid degeneration of collagen and elastin fibres, with hydropic degeneration of the epidermis and dissociation of epithelial glandular and endothelial cells, but also to loss of striation of muscle cells and of their normal staining capacity, with subsequent myolysis and haemorrhage. There was no change in the nuclei.

The presence of a collagenase in *Cl. histolyticum* cultures was first recorded by Jennison (1945) who showed that they disintegrated heated *tendo Achillis* of cow, and also Highberger's purified steer-hide collagen sterilized by heating; their activity was enhanced by thiolacetate. Later (1947), Jennison provided more convincing evidence by showing that filtrates attacked fine strands of clean unprocessed beef tendon, whether it was sterilized by dry heat or not; he followed the process of digestion by determining the loss of weight in the collagen used. Evans (1947) observed that *Cl. histolyticum* produced a substance which attacked hide-powder and was distinct from the lethal toxin. This substance might be a collagenase or one of the proteinases that attacks degraded collagen.

EXPERIMENTAL

Cultural methods and production of toxins. Eight strains of *Cl. histolyticum* were obtained as desiccates from the Culture Collection of these Laboratories. It seems reasonably certain that five of these are distinct strains; no evidence can be provided to support the distinctness of the others. Smooth colonies on agar plates were selected from all the strains and grown in 50 ml. quantities of Brewer's medium for 24 hr., or in larger volumes of nutrient broth or a papain digest of horse muscle for 16–24 hr. The cultures were largely freed from bacteria by the addition of 'Hyflo' Supercel (Johns, Manville; 5 g./l.) and filtration through paper, and if necessary sterilized by candling. In general, the methods were similar to those found satisfactory for producing the toxic culture filtrates of *Cl. oedematiens* (Oakley, Warrack & Clarke, 1947).

Sera. We had only six sera, produced some years before by Mr A. T. Glenny, F.R.S., by immunizing horses with toxic culture-filtrates of *Cl. histolyticum* and with toxoids made from them. Testing for antibodies was carried out by conventional methods, using serum RR 2001 (100 units) as standard.

Examination of culture-filtrates for toxins

Lethal toxins. Filtrate + serum mixtures were allowed to stand for 30 min. at room temperature, then injected intravenously in 0·5 ml. volumes into mice weighing 16–20 g. Level of test, 0·25, 0·5 or 1 unit injected, according to strength of filtrate; standard indicating effect, death within 48 hr. of half the mice injected.

Table 1. *Values of sera in lethal tests against* Clostridium histolyticum *filtrates*

Serum	Serum values against filtrate		
	CN 647	CN 919	CN 949
RR 2001	100	100	100
RR 2005	230	240	260
RR 2008	25	30	25
RR 2035	530	570	600
RR 8003	55	50	60
RR 8006	70	75	75

Only three filtrates were lethal in sufficiently small amounts to make reasonably accurate testing possible. Table 1 shows that the serum values in lethal tests were, within the rather considerable experimental error, the same whichever filtrate was used. The lethal toxin for which the end-point was determined was therefore probably the same for all three filtrates; there was no evidence of a second lethal antigen.

Necrotizing toxins. Filtrate + serum mixtures were allowed to stand for 30 min. at room temperature, then injected intracutaneously in volumes of 0·2 ml. into guinea-pigs. Level of test 0·2, 0·4 or 0·8 unit injected, according to strength of filtrate; standard indicating effect, a small necrotic lesion. (In under-neutralized mixtures a characteristic circular oedematous purple necrotic lesion was produced.)

Table 2. *Comparison of anti-lethal and anti-necrotizing values of sera, to show that the lethal toxin of* Clostridium histolyticum *has necrotizing properties*

Serum	Serum values in lethal tests	Serum values in necrotizing tests against filtrate		
		CN 647	CN 919	CN 949
RR 2001	100	100	100	100
RR 2005	240	240	250	250
RR 2008	27	25	24	25
RR 2035	570	550	550	530
RR 8003	55	55	52	46
RR 8006	73	65	65	60

Table 2 shows that serum values in necrotizing tests against all the three filtrates used were constant for any particular serum and equal to the anti-lethal values; thus the lethal toxin is also necrotizing.

Collagenases. Filtrate + serum mixtures were allowed to stand at room temperature for 30 min. in tubes 6 cm. long × 0·8 cm. internal diameter (Lambeth tubes); they were then incubated overnight with pieces of fresh guinea-pig muscle or collagen paper (Oakley, Warrack & van Heyningen, 1946; Delaunay, Guillaumie & Delaunay, 1949). Level of test 1 unit; standard indicating effect: a well-marked softening of muscle or disintegration of collagen paper.

Table 3. *Comparison of anti-lethal values of sera with those obtained in muscle-softening and collagen paper tests to show that the collagenase* (β) *is distinct from the lethal toxin* (α)

		Serum values against filtrate					
		NX 729 using as indicator		NX 730 using as indicator		CN 647 using as indicator	
Serum	Serum values in lethal tests	Fresh muscle	Collagen paper	Fresh muscle	Collagen paper	Fresh muscle	Collagen paper
RR 2001	100	100	100	100	100	100	100
RR 2005	240	330	350	320	250	310	350
RR 2008	27	370	370	330	370	320	370
RR 2035	570	220	240	200	210	250	230
RR 8003	55	180	180	160	200	—	260
RR 8006	73	170	170	170	160	150	180

Table 3 shows that the serum values against collagen paper or guinea-pig muscle as indicators are independent of the filtrate used and agree with one another, though they bear no relationship to the values obtained in lethal tests. The collagenase (β) is therefore distinct from the lethal and necrotizing toxin (α).

Oakley, Warrack & Warren (1948) showed that *Cl. welchii* collagenase (κ-toxin) has lethal and necrotizing activities; unfortunately the very low anti-lethal values of our *Cl. histolyticum* antisera, as compared with their anti-collagenase values, have made it impossible so far to decide whether *Cl. histolyticum* collagenase is lethal and necrotizing.

All the *Cl. histolyticum* antisera contained small amounts of *Cl. welchii* κ-antitoxin; comparison of the *Cl. welchii* anti-κ values with the *Cl. histolyticum* anti-β values shows that there is no relationship between them; the collagenases of *Cl. welchii* and *Cl. histolyticum* are antigenically distinct.

Other proteinases. Filtrate serum mixtures were allowed to stand for 30 min. at room temperature, and were then incubated overnight in a water-bath at 37° with 'azocoll' (Oakley *et al.* 1946); this material is attacked by several proteolytic enzymes which do not attack collagen (Todd, 1947; Oakley *et al.* 1948). Level of test one unit; standard indicating effect, diffusion of a small amount of colour from the azocoll.

Table 4 shows that serum values against azocoll as indicator are divisible into two groups, those corresponding to the anti-β values, and those that are much lower. The lower values are characteristic of two sera RR 2005 and RR 2035, and it is noticeable that though their values differ according to the filtrate used for test, the values for the two sera are substantially equal against any given

Table 4. *Comparison of anti-collagenase values of sera and those found in azocoll tests, to show evidence of an enzyme (γ) attacking azocoll but not collagen*

Serum	Anti-collagenase values*	Serum values in azocoll tests against filtrate			
		CN 647*	CN 919*	NX 729†	NX 730†
RR 2001	100	100	100	100	100
RR 2005	350	160	160	140	140
RR 2008	380	370	450	300	250
RR 2035	250	160	210	150	170
RR 8003	210	320	230	225	210
RR 8006	160	190	180	190	190

* All the values in these columns were obtained in 1946.
† Values in these columns obtained in 1949.

filtrate. In other words, the end-point given by these two sera in azocoll tests is for a proteinase (γ) capable of attacking azocoll but not collagen; the anti-γ values of RR 2005 and RR 2035 are approximately equal, their anti-β values are not.

Addition of 0·05 M cysteine to one of the culture filtrates (from *Cl. histolyticum* CN 647) had no effect on its test dose or on its standard indicating dose against collagen paper; its activity against azocoll was slightly increased, and its test dose against this material was diminished to about one-half, using serum RR 2001 as standard. As the test dose against collagen paper was unaffected by addition of cysteine, one may reasonably assume that the collagenase test dose against azocoll was unaffected also; therefore, since the test dose of the cysteine activated filtrate was diminished to one-half in azocoll tests, any serum giving the collagenase end-point would appear to double in value when tested against it. Table 5 shows that sera RR 8003 and RR 8006

Table 5. *Comparison of anti-collagenase values of sera with those obtained in azocoll tests, to give further evidence of the existence of γ, and of its activation by cysteine. The relevant end-points are indicated in brackets*

Serum	Anti-collagenase values	Serum values in azocoll tests against filtrate CN 647		
		Unmodified	+0·05 M cysteine	+0·01 M iodoacetate
RR 2001	100	100 (β or γ)	100 (γ)	100 (β or γ)
RR 2005	320	160 (γ)	160 (γ)	150 (γ)
RR 2008	360	370 (β or γ)	360 (γ)	345 (β)
RR 2035	225	160 (γ)	180 (γ)	140 (γ)
RR 8003	260	320 (β)	550 (β)	290 (β)
RR 8006	180	190 (β)	410 (β)	190 (β)

have apparently doubled their values when tested against cysteine-activated filtrate in azocoll tests; thus they are giving the collagenase (β) end-point. When all the serum values are divided by two, so that the values of RR 8003 and RR 8006 are decreased to their usual anti-β values, all the other sera show values less than their anti-β values; their end-points are thus clearly for an antigen other than β. It cannot certainly be stated what this antigen is, but

since sera RR 2005 and RR 2035 give equal values against cysteine-activated filtrate, it is probable that the end-point given by sera other than RR 8003 and RR 8006 is the γ-end-point, and that γ-antigen is activated by cysteine. To look at it in another way, if before activation with cysteine the $\beta:\gamma$-antigen ratio in CN 647 was 1:1, after activation it was 1:2, with corresponding effects on the values of sera titrated against it (see Oakley, 1943).

It follows, moreover, that if the standard serum RR 2001 is given an anti-γ value of 100 units, the anti-γ values of the remaining sera will be those found in azocoll tests against cysteine-activated culture filtrate of CN 647, except for RR 8003 and RR 8006, whose anti-γ values will be greater than 550 units and greater than 410 units respectively.

For filtrate CN 647 neither the test dose against collagen paper nor the test dose against azocoll was affected by 0·01 M iodoacetate; presumably neither β nor γ-antigen is activated or inhibited by it. Serum values against iodoacetate-treated filtrate did not differ from those against untreated filtrate. This filtrate therefore appeared to contain an enzyme (γ) similar to the cysteine-activated proteinase described by Weil & Kocholaty (1937) and by Maschmann (1938); no evidence has been obtained of enzymes inhibited by cysteine or iodoacetic acid; we hope to carry out a more thorough examination of filtrates for such proteinases in the near future.

Gelatinases. Serum values were determined in parallel in azocoll and gelatin-agar tests (Oakley *et al.* 1948) against two filtrates NX 729 and NX 730. Level of test in gelatin agar tests 1 unit; standard indicating effect, a clear zone of convenient size. The serum values (Table 6) show that in azocoll tests these

Table 6. *Comparison of anti-β and anti-γ values of sera with those obtained in azocoll and gelatin-agar tests to show that both β and γ attack azocoll and gelatin*

			Serum values against filtrate			
			NX 729 using as indicator		NX 730 using as indicator	
Serum	Anti-β values	Anti-γ values	Azocoll	Gelatin-agar	Azocoll	Gelatin-agar
RR 2001	100	100	100	100 (β or γ)	100	100
RR 2005	320	160	140	170 (γ)	140	160
RR 2008	360	360	300	360 (β)	250	350
RR 2035	225	150	150	160 (γ)	130	170
RR 8003	260	>550	225	240 (β)	210	270
RR 8006	180	>410	190	230 (β)	190	230

filtrates behaved very much like the previous non-activated filtrates: RR 2005 and RR 2035 gave the γ-end-point, RR 8003 and RR 8006 the β-end-point, while RR 2001 and RR 2008 gave either the β or γ-end-point. Serum values in gelatin agar tests were, as compared with the standard, slightly higher than the values in azocoll tests. The difference was small, and will need much more work to confirm. In general the results are of the same order in gelatin-agar and in azocoll tests, and the simplest explanation is that the same end-point is determined in both. If this is so, both β and γ attack gelatin.

We wish to express our very grateful thanks to Mrs Patricia Clarke for much bacteriological assistance, to her and to Miss H. E. Ross for producing numerous toxic culture-filtrates, and to Miss Marian E. Warren for considerable technical assistance.

REFERENCES

Blanc, J. & Pozerski, E. (1920*a*). Sur les ferments protéolytiques de quelques anaérobies pathogènes. *C.R. Soc. Biol., Paris*, **83**, 1343.

Blanc, J. & Pozerski, E. (1920*b*). Sur les ferments protéolytiques du *B. sporogenes* et du *B. histolyticus*. *C.R. Soc. Biol., Paris*, **83**, 1369.

Beckwith, T. D. & MacKillop, G. (1923–4). Effects produced by injection of *B. histolyticus*. *J. med. Res.* **44**, 311.

Bessey, O. & King, C. G. (1934). Proteolytic and deaminising enzymes of *Cl. sporogenes* and *Cl. histolyticum*. *J. infect. Dis.* **54**, 123.

Carlen, S. A. (1939). Fibrinolytic properties of filtrates of *Cl. histolyticum* in normal saline and dog serum. *Proc. Soc. exp. Biol., N.Y.*, **40**, 39.

Delaunay, M., Guillaumie, M. & Delaunay, A. (1949). A propos des collagénases bacteriennes. *Ann. Inst. Pasteur*, **76**, 16.

Evans, D. G. (1947). The production by certain species of *Clostridium* of enzymes disintegrating hide-powder. *J. gen. Microbiol.* **1**, 378.

Gildemeister, E. & Schlossberger, H. (1932). Ueber die Wirkung des Toxins der Erreger der Gasbrandgruppe auf die Kaninchenhornhaut. *Zbl. Bakt.* (1, Abt., Orig.), **126**, 527.

Hall, I. C. (1922–3). The failure of fermentation reactions with *B. histolyticus*. *Proc. Soc. exp. Biol., N.Y.*, **20**, 503.

Hall, I. C. (1923–4). Recovery of *B. histolyticus* from human faeces. *Proc. Soc. exp. Biol., N.Y.*, **21**, 198.

Hall, I. C. & Peterson, E. (1922–3). A note on the mechanism of the peculiar lesions produced by *Bacillus histolyticus*. *Proc. Soc. exp. Biol., N.Y.*, **20**, 503.

Heyningen, W. E. van (1940). The proteinases of *Cl. histolyticum*. *Biochem. J.* **34**, 1540.

Hoogerheide, J. C. (1937). Variability in morphological and biochemical properties of *Cl. histolyticum* (Weinberg & Séguin). *J. Bact.* **34**, 387.

Jennison, M. W. (1945). Bacterial collagenase. *J. Bact.* **50**, 369.

Jennison, M. W. (1947). The collagenase activity of culture filtrates of *Cl. histolyticum*. *J. Bact.* **54**, 55.

Kahn, M. C. (1922). A cultural study of anaerobic spore-bearing bacteria with strains isolated by the Barber single-cell technique. *J. med. Res.* **43**, 155.

Kocholaty, W. & Krejci, L. E. (1948). The activation mechanism and physico-chemical properties of *Cl. histolyticum* proteinase. *Arch. Biochem.* **18**, 1.

Kocholaty, W. & Weil, L. (1938). Enzymic adaptation in *Cl. histolyticum*. *Biochem. J.* **32**, 1696.

Kocholaty, W., Weil, L. & Smith, L. (1938). Proteinase secretion and growth of *Cl. histolyticum*. *Biochem. J.* **32**, 1685.

Maschmann, E. (1938). Ueber Bakterienproteasen. IV. Mitteilung. Die Proteasen des *B. histolyticus*. *Biochem. Z.* **295**, 391.

Nasta, M. (1922). Contribution à l'étude de l'action du *B. histolyticus* sur les tissus. *C.R. Soc. Biol., Paris*, **87**, 279.

Oakley, C. L. (1943). The toxins of *Cl. welchii*. A critical review. *Bull. Hyg., Lond.*, **18**, 781.

Oakley, C. L., Warrack, G. H. & Clarke, P. H. (1947). The toxins of *Cl. oedematiens* (*Cl. novyi*). *J. gen. Microbiol.* **1**, 91.

Oakley, C. L., Warrack, G. H. & Heyningen, W. E. van (1946). The collagenase (κ-toxin) of *Cl. welchii* type A. *J. Path. Bact.* **58**, 229.

Oakley, C. L., Warrack, G. H. & Warren, M. E. (1948). The kappa and lambda antigens of *Cl. welchii*. *J. Path. Bact.* **60**, 495.

Pasternack, J. G. (1940). The experimental pathological changes produced by the toxin of *Cl. histolyticum* in animals. *Publ. Hlth Rep., Wash.*, **55**, 775.

Peterson, E. & Hall, I. C. (1922–3). The isolation of *B. histolyticum* from soil in California. *Proc. Soc. exp. Biol., N.Y.*, **20**, 502.

Pozerski, E. & Guélin, A. (1938*a*). Action empêchante de l'ovalbumine crue sur les ferments gélatinolytiques de quelques microbes anaérobies. *C.R. Soc. Biol., Paris*, **128**, 504.

Pozerski, E. & Guélin, A. (1938*b*). Contribution à l'étude des filtrats de quelques microbes anaérobies. Gelatinase et pouvoir escarrifiant. *C.R. Soc. Biol., Paris*, **128**, 842.

Reed, G. B., Orr, J. H. & Brown, H. J. (1943). Fibrinolysins from gas gangrene anaerobes. *J. Bact.* **46**, 475.

Todd, E. W. (1947). A study of the inhibition of streptococcal proteinase by sera of normal and immune animals and of patients infected with group A haemolytic streptococci. *J. exp. Med.* **85**, 591.

Torrey, J. C. (1925). Habitat of *B. histolyticus* in human intestine. *J. infect. Dis.* **36**, 517.

Weil, L. & Kocholaty, W. (1937). Studies on proteinase of *Cl. histolyticum*. *Biochem. J.* **31**, 1255.

Weinberg, M. & Séguin, P. (1916). Contribution à l'étiologie de la gangrène gazeuse. *C.R. Acad. Sci., Paris*, **163**, 449.

(*Received* 14 *January* 1950)

Some Factors Influencing the Rate of Formation of Tetrathionase

By W. H. H. JEBB, R. KNOX and A. H. TOMLINSON

Public Health Laboratory, Oxford

SUMMARY: The intermediate type I coliform organism 1433 can use, as hydrogen donators during the adaptive formation of the tetrathionase enzyme system, most of the carbohydrates that it can ferment, although in some cases the cells must first be grown on a medium containing the carbohydrate concerned. Not only the reduction of tetrathionate, but the process of adaptive formation of tetrathionase as well, is inhibited by oxygen gas, but the capacity to adapt when favourable conditions are restored is relatively unaffected by oxygen. The amount and the rate of formation of new enzyme are greatly increased by providing a source of available nitrogen. The rate of formation is also increased by incubating the cells with mannitol and phosphate before tetrathionate is added, but the total amount of enzyme formed is not increased: it is probable that the increased rate is due to the accumulation of intermediates within the cells; these intermediates have not so far been identified. None of the mathematical expressions investigated fits the course of tetrathionase adaptation exactly; that put forward by Spiegelman (1945) for the production of yeast galactozymase approximates most closely.

Knox & Pollock (1944) described in the *Salmonella* and *Proteus* groups and in certain coliform organisms an adaptive enzyme system which reduced tetrathionate quantitatively to thiosulphate. This adaptation occurred in washed suspension, in the absence of a nitrogen source and without demonstrable increase in cell numbers or cell material. The effect of certain alterations in the composition of the suspending fluid was also investigated, and Pollock (1945) described the effect of temperature on tetrathionase adaptation.

Gale (1943) reviewed the factors influencing the activity of bacterial enzymes, and Monod (1947) described the particular characteristics of 'adaptation', which he defined as 'apo-enzyme formation induced by a specific substrate'. The evidence for the production of a new enzyme is often largely circumstantial, but in the case of yeast galactozymase various workers (Kosterlitz, 1943; Spiegelman, Reiner & Morgan, 1947; Wilkinson, 1949) have presented convincing evidence of the presence of a new enzyme in the adapted cells. The results of the present investigation are interpreted on the assumption that new enzyme is formed; the evidence for this will be discussed later.

In this investigation an attempt was made to find a mathematical expression for the rate of enzyme production and to test its agreement with Spiegelman's theory of enzyme formation (Spiegelman, 1945). In addition, the effects of oxygenation, an added nitrogen source, and pre-incubation of the cells with mannitol and phosphate, have been studied in an attempt to reach a fuller understanding of the adaptive process.

METHODS

Organism. The organism used was the coliform intermediate type I labelled '1433' used by Knox, Gell & Pollock (1943) in their work on the selective action of tetrathionate, and subsequently by Pollock (1946), Pollock & Wainwright (1948) and Jebb (1949).

Preparation of suspensions. All suspensions were prepared by inoculating 1 ml. of a 6 hr. tryptic heart broth culture on to 200 ml. tryptic heart agar in Roux bottles. The Roux bottles were incubated at 37° for 15–17 hr. and the cells washed off with distilled water. The cell suspension was centrifuged, the cells washed twice with distilled water and made into a thick suspension in water. The cell suspension was adjusted, using a Hilger Biochem Absorptiometer, to contain 12–14 mg. dry weight/ml.; this was the stock cell suspension.

Adaptation experiments. Unless otherwise stated, the following final concentrations were used in adaptation reaction mixtures: stock cell suspension diluted 1/5; phosphate buffer (pH 7·6), 0·2M; mannitol, 0·02M; sodium tetrathionate, 0·02M. Samples were removed at intervals and mixed with twice their own volume of 10 % (v/v) aqueous acetic acid to which a few drops of 0·5 % starch were added, and titrated with 0·005N iodine.

Sodium tetrathionate was prepared by the method described by Gilman, Phillips, Koelle, Allen & St John (1946).

Temperature. All the experiments were carried out at 37°.

RESULTS

The nature of the hydrogen donator

Of the common 'sugars' used in diagnostic bacteriology the organism 1433 ferments glucose, fructose, galactose, maltose, lactose, arabinose, xylose, sorbitol, mannitol and glycerol. With the exception of xylose, all of these acted as hydrogen donators for rapid adaptation, though galactose, lactose, maltose, arabinose and sorbitol could be so utilized only by cells previously grown on media containing the compound concerned. The enzymes attacking these latter compounds are presumably adaptive.

Other substances which could be dehydrogenated by the enzymes of the cell, namely lactate, formate, succinate and ethanol, could supply hydrogen for reduction of tetrathionate by adapted cells, but did not stimulate a rapid adaptation of fresh cells. Hence the hydrogen donator for rapid adaptation in washed suspension must be fermentable.

Measurement of tetrathionase activity

To study the adaptive process in detail it was desirable to determine the tetrathionase activity at various times during the process. This involved measuring the rate of reduction of tetrathionate by partially adapted cells under conditions where further adaptation could not occur. This could be done by using formate or lactate as hydrogen donator, or by inhibiting adaptation in the presence of mannitol by 2:4-dinitrophenol or sodium azide.

The choice of a suitable method presented some difficulty. Table 1 shows that the activity of tetrathionase as measured by the rate of reduction was dependent on the hydrogen donator used and also on the activity of the dehydrogenating system—as shown by the variation in relative rates with different H donators in different experiments. The stimulation of reduction by the dinitrophenol appears similar to its effect on the oxygen uptake of *Bact. coli* (Clifton, 1946) and of frog muscle (Ronzoni & Ehrenfest, 1936).

Table 1. *Rates of reduction of tetrathionate by fully adapted cell suspensions of organism* 1433 *in the presence of various hydrogen donators*

(Tubes contained (final concentrations): adapted cell suspension 1/10, phosphate buffer (pH 7·6) 0·2 M, sodium tetrathionate 0·02 M and the hydrogen donator in the concentration indicated.)

Hydrogen donator or other addendum (final concentration)	Tetrathionase activity (μmol. thiosulphate produced/mg. dry wt./hr.)		
	Exp. 1	Exp. 2	Exp. 3
Mannitol, 0·02 M; Dinitrophenol, 0·0005 M	18·6	31·8	—
Mannitol, 0·02 M	17·7	25·9	19·3
Sodium lactate, 0·02 M; Sodium formate, 0·02 M	17·2	29·0	25·1
Sodium lactate, 0·02 M	16·8	25·4	18·9
Sodium formate, 0·02 M	6·4	10·4	15·1

Fig. 1 shows the rates of reduction of tetrathionate, as determined with different hydrogen donators, at different stages of adaptation. From the figure it can be seen that mannitol with dinitrophenol gave the highest rate, but this combination was considered unsuitable because the graph crossed the lactate curve, suggesting that the dinitrophenol stimulation increased with time of adaptation. Formate was not considered suitable because during adaptation in the presence of heart broth formic hydrogenlyase was produced (as shown by Warburg respirometer experiments) and, while this may be irrelevant, it was thought wiser to avoid unnecessary complications. Organism 1433 possesses a hydrogenase, and gaseous hydrogen, in the Warburg respirometer, was tried as a hydrogen donator but proved unsatisfactory because of the high blank reduction. The hydrogen uptake accounted for only 60 % of the tetrathionate reduced. Sodium lactate was eventually chosen as a hydrogen donator because it appeared to reveal a high proportion of the tetrathionase activity of all suspensions and it was not open to the above objections.

The method finally adopted for estimating the tetrathionase activity, which we define as the rate of reduction under standard conditions, was the following:

10 ml. samples were removed from the adaptation mixtures, cooled rapidly, centrifuged, washed twice in distilled water, suspended in a small volume of water and added to 2 ml. M phosphate buffer (pH 7·6), 1 ml. sodium lactate 0·5 M, and 1 ml. sodium tetrathionate 0·2 M, and made up to a volume of 10 ml. Samples were titrated with iodine as described previously, and the tetrathionase activity expressed in terms of μmol. thiosulphate produced/mg. dry wt. cells/hr.

Adaptation under standard conditions

The course of tetrathionate reduction by adapting cells is shown in Fig. 2. The increase of activity with time is shown by curve (*a*) of Fig. 3. To facilitate comparison of adaptations under different conditions it seemed desirable to obtain an equation which would represent the adaptation process, and provide a measure of the rate of enzyme production.

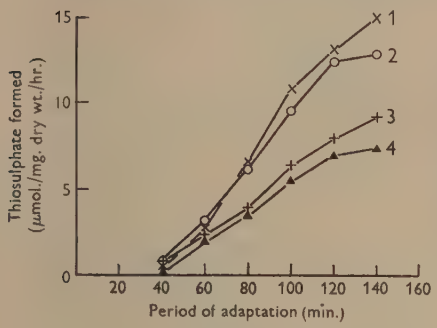


Fig. 1

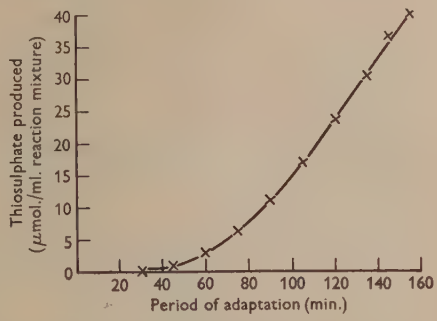


Fig. 2

Fig. 1. Tetrathionase activities determined with different hydrogen donators after various periods of adaptation in standard reaction mixture. Each assay tube contained: 2 ml. cell suspension; 2 ml. M phosphate (pH 7·6); 1 ml. 0·2M sodium tetrathionate; and in curve 1 (×——×), 1 ml. 0·2M mannitol, 0·5 ml. 0·01M 2:4-dinitrophenol and 3·5 ml. water; in curve 2 (○——○), 1 ml. 0·5M sodium lactate and 4 ml. water; in curve 3 (+——+), 1 ml. 0·2M sodium formate and 4 ml. water; in curve 4 (▲——▲), 1 ml. 0·4 methanol and 4 ml. water.

Fig. 2. Tetrathionase adaptation under standard conditions. Reaction mixture contained: stock cell suspension diluted 1/5; phosphate buffer (pH 7·6), 0·2M; mannitol, 0·02M; sodium tetrathionate, 0·02M. Samples were removed at intervals and the thiosulphate produced titrated with 0·005N iodine.

From Fig. 2 it appeared that the process might be represented either by an exponential function or by an equation such as

$$\text{thiosulphate formed} = \text{constant} \times (\text{time})^n,$$

where n is 2 or 3. A plot of log (thiosulphate formed) against time was linear only during the early stages of adaptation. Graphs of thiosulphate formed against the square of time and against the cube of time were each linear during a part of the process, but neither gave agreement over the whole period of adaptation, nor could any of these expressions account for the gradual cessation of adaptation.

Spiegelman (1945) found that the galactozymase adaptation of yeast was adequately represented by the equation

$$E = \frac{\overline{P}}{1 + e^{a - \overline{k}t}},$$

where E is the amount of enzyme present at time t, $\overline{P}$ is the amount of enzyme

finally formed from a precursor P, and a and k are constants. This equation can be rearranged

$$a-kt=\ln\frac{\overline{P}-E}{E},$$

and the fit of a set of points can be tested by plotting $\log\frac{\overline{P}-E}{E}$ against t.

Fig. 4 shows that between 40 and 120 min., the period during which most of the tetrathionase adaptation occurred, the points obtained lay on a straight line, but the point for zero time consistently fell below this line. This dis-

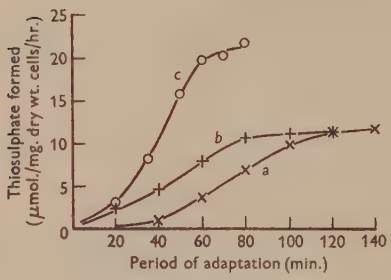


Fig. 3

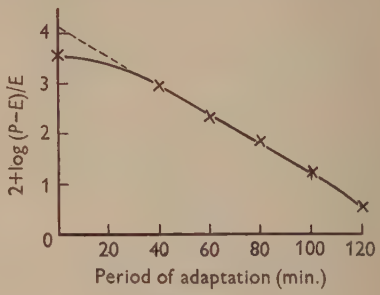


Fig. 4

Fig. 3. Tetrathionase activity during adaptation in solutions of the various compositions described: 20 ml. stock cell suspension; 20 ml. M phosphate (pH 7·6); 10 ml. 0·2M mannitol; 10 ml. 0·2M sodium tetrathionate (all throughout). In addition, in curve *a* (×——×), +40 ml. water; curve *b* (+——+), + 40 ml. water but in this tube the 10 ml. 0·2M sodium tetrathionate was not added until the rest of the mixture had been incubated for 60 min.; curve *c* (○——○), +40 ml. tryptic heart broth.

Fig. 4. Spiegelman's (1945) formula applied to tetrathionase adaptation. Data calculated from Fig. 3, curve *a*.

crepancy might be due to the determined value of the initial enzyme content being too high, or could be accounted for by a lag during which no adaptation occurred. In a number of experiments the zero-time point Z was regularly 17–20 min. to the left of the line.

Changing the conditions of adaptation (as described later) altered the shape of the $\log \overline{P}-E/E$ time curve, but not in a manner sufficiently consistent to be of any value in comparing adaptations, and it was necessary to fall back on visual comparisons of curves of the type shown in Fig. 3.

It is perhaps relevant to point out that considerable caution is necessary in applying simple mathematical treatments to complex biological phenomena; Monod (1942), for example, has stressed the dangers of such a procedure.

Effect of oxygen on adaptation

Pollock & Knox (1943) showed that oxygenation virtually inhibited reduction of tetrathionate by *Salmonella paratyphi* B, an effect analogous to that of oxygen on nitrate reduction described by Stickland (1931).

Oxygen also inhibits *adaptation* to tetrathionate reduction. Cells were incubated for 145 min. (*a*) with mannitol, phosphate buffer and tetrathionate; (*b*) with mannitol, phosphate buffer and tetrathionate and oxygen bubbled

through continuously; (*c*) diluted with water to the same dilution as (*a*) and (*b*). The cells were then centrifuged off, washed and assayed for tetrathionase. The un-oxygenated cells (*a*) produced 11·9 μmol. thiosulphate/mg./hr., whereas the oxygenated cells (*b*) and the control cells (*c*) produced 0·24 μmol. thiosulphate/mg./hr.

Oxygenation also 'de-adapts' previously adapted cells. Fully adapted cells suspended in 0·05 M phosphate buffer were oxygenated during incubation at 37° and at intervals samples were taken out and assayed. Fig. 5 shows the

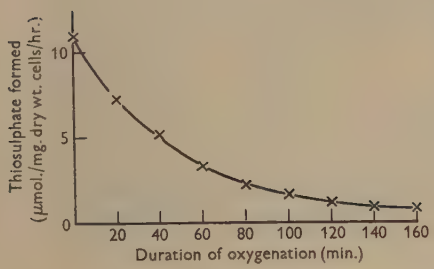


Fig. 5

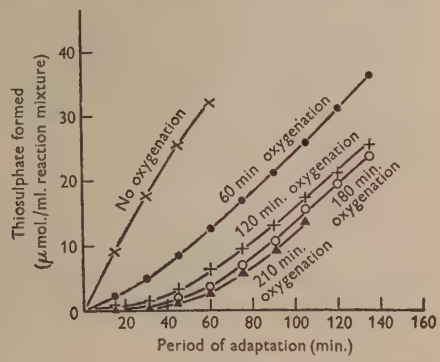


Fig. 6

Fig. 5. Effect of oxygenation on the tetrathionase activity of adapted cells of organism 1433.

Fig. 6. Re-adaptation of previously adapted cells 'de-adapted' by oxygenation for various times. Cells, after oxygenation for various periods (as shown on diagram), were added to a reaction mixture to give the following final concentrations: stock cell suspension diluted 1/5; phosphate buffer (pH 7·6), 0·2 M; mannitol, 0·02 M; sodium tetrathionate, 0·02 M.

activities of the samples plotted against time of oxygenation. Other samples were put into adaptation mixtures of mannitol, tetrathionate and buffer. From Fig. 6 it can be seen that oxygenation effected a reversible 'de-adaptation', leaving the ability to adapt relatively unimpaired.

Attempts to reactivate such de-adapted cells by incubation in reducing agents such as ascorbic acid, thiolacetate and glutathione were unsuccessful. On the other hand, the presence of tetrathionate during oxygenation did have a slight protective effect and retarded the de-adaptation.

The effect of an added nitrogen source

Pollock & Wainwright (1948) showed that the addition of a mixture of amino-acids increased the rate of nitratase adaptation in washed suspensions of the coliform organism 1433. The course of tetrathionase adaptation is markedly changed by supplying to the adapting cells a source of readily available nitrogen. Fig. 3 (curve *c*) shows that when 40 % (v/v) of tryptic heart broth was included in the reaction mixture enzyme was produced faster and reached a final activity almost twice that of the same cells without broth (curve *a*)—an exact parallel to Spiegelman's (1945) observation with yeast

galactozymase. During adaptation in broth the optical density of the cell suspension increased by about 10 % (of which some could be accounted for by the precipitated sulphur known to be present) but total and viable cell counts showed no increase.

A similar stimulation was produced by casein digest, yeast extract, liver extract and peptone water. A comparison between the following stimulating substances was made by including them in adaptation mixtures namely (*a*) the laboratory tryptic heart broth; (*b*) tryptic digest of casein prepared according to Gladstone & Fildes (1940); (*c*) an extract prepared by steaming 90 g. minced ox-liver with 30 ml. water for 1 hr., filtering and adjusting to pH 7·6. The total nitrogen contents of these solutions were determined by the Kjeldahl method and each was then diluted to contain 1·7 mg. N/ml. These solutions were included in adaptation mixtures of the composition shown in Table 2 which records the results.

Table 2. *Effect of different nitrogen sources on the adaptation of organism* 1433

(The added nitrogen sources each contained 1·7 mg. N/ml. and were added in volumes of 20 ml. to the following adaptation mixtures: cell suspension, 10 ml.; phosphate buffer (M), 10 ml.; mannitol, 0·2 M, 5 ml.; tetrathionate, 0·2 M, 5 ml.; and nitrogen source, 20 ml. (i.e. 40 % of the total volume). At intervals samples of the cells were assayed for tetrathionase activity.)

Time of sampling (min.)	Added nitrogen source: None	Liver extract	Broth	Casein digest
	Tetrathionase activity (μmol. thiosulphate produced/mg. dry wt./hr.)			
30	0·2	13·5	2·5	2·3
50	1·0	23·8	11·3	2·7
70	2·7	29·5	18·7	16·8

From the rapidity of adaptation in presence of liver extract it seemed probable that the solution contained specific stimulatory factors. As the optical density of the suspensions increased by 20 % during the adaptation, growth might have been beginning, but total and viable counts did not indicate any significant cell division.

Pre-treatment of cells with mannitol and phosphate

When cells were incubated at 37° with mannitol and buffer before tetrathionate was added, the rate of adaptation was increased without affecting the final amount of enzyme produced (Fig. 3, curve *b*) in contrast with the effect of an added nitrogen source. This acceleration was produced by pre-treatment with mannitol, glucose or fructose but not with lactate, formate or phosphate alone. The extent of the acceleration depended on the duration of pre-treatment; maximum acceleration was produced by 60–90 min. pre-treatment. It can be seen from Fig. 7 that the linear portions were parallel, that is, the final enzyme activities were equal, but the time taken to reach this rate decreased as the duration of pre-treatment increased.

Of the possible explanations of this acceleration, the following seemed the most probable: (1) adaptation to mannitol fermentation, which experiments in the Warburg respirometer and on methylene-blue reduction times did not support (see also Pollock, 1946); and (2) the accumulation of some metabolic intermediate or intermediates necessary for adaptation. This postulated intermediate must be in the cells or in the suspending fluid or both, and investigations were carried out in an attempt to determine its location.

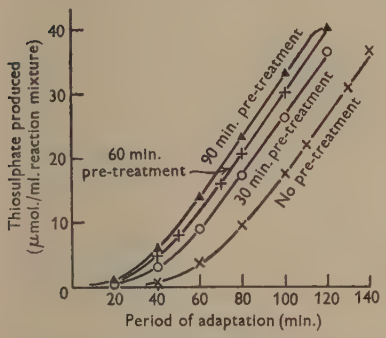


Fig. 7

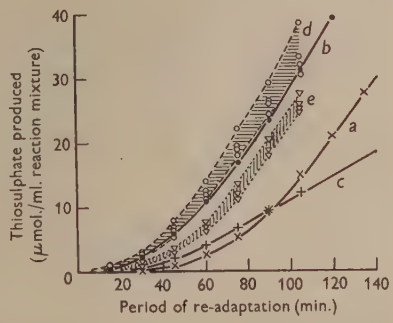


Fig. 8

Fig. 7. Effect of duration of pre-treatment with mannitol and phosphate on the adaptation of washed cells of organism 1433. Each tube contained: stock cell suspension diluted 1/5; phosphate buffer, 0·2 M; mannitol, 0·02 M; tetrathionate, 0·02 M. The tetrathionate was added after the periods of pre-treatment indicated and at intervals samples were removed from each for titration with iodine.

Fig. 8. The change produced in the cells of organism 1433 by pre-treatment with mannitol and phosphate, and the non-specific nature of the changes occurring in the suspending fluid. Curve *a* (×——×), standard (untreated) adaptation for comparison; curve *b* (●——●), adaptation after pre-treatment for comparison; curve *c* (+——+), pre-treated 1433 cells added to fresh reaction mixture; curves *d* (○ - - - - ○), pre-treated 1433 cells added to supernatant fluids from incubation of 1433 or *Bact. coli* cells with mannitol and phosphate or phosphate only; curves *e* (▽ . . . . ▽), untreated 1433 cells added to supernatant fluids from incubation of 1433 or *Bact. coli* cells with mannitol and phosphate or phosphate only.

Pre-treated cells were centrifuged off from their suspending fluid and re-suspended in fresh mannitol, phosphate and tetrathionate (Fig. 8, curve *c*). Apart from some early stimulation of adaptation they were found to be even less active than untreated cells added to the same mixture (Fig. 8, curve *a*). When the cells were washed before they were added to the new reaction mixture they showed even less activity.

The loss of activity on transferring pre-treated cells to a new reaction mixture was not due to aeration during the manipulations involved, because when, instead of centrifugation, the mixture was oxygenated at room temperature for 30 min. after pre-treatment, it still showed its full activity on addition of tetrathionate. Nor was it likely to be due to the metabolism of an intermediate, since the activity of pre-treated cells was still lost even if the cells were chilled to 4° and kept cold during centrifugation and washing. The activity

lost after centrifugation was, however, fully regained by resuspending the cells in their own supernatant fluid.

The suspending fluid. These observations suggested that some material essential for adaptation was being leached out of the cells by the suspending fluid, and that the inactivation which occurred when the supernatant fluid was discarded was due to loss of this material. The following experiment was performed to investigate the effect of phosphate buffer on the cells.

Two lots of unadapted cells were incubated in 0·2 M buffer for 60 min. and then treated as follows:

(i) Mannitol and tetrathionate were added. It was found that adaptation was slightly less rapid (Fig. 9, curve *b*) than that of fresh, untreated, cells put up to adapt in the ordinary way (Fig. 9, curve *a*).

(ii) The cells were centrifuged off from the buffer and resuspended in fresh reaction mixture. These cells showed very little activity (Fig. 9, curve *c*), whereas the buffer in which they had been incubated, when added to untreated cells, stimulated their adaptation (Fig. 9, curve *d*).

It can thus be seen that the power of cells to adapt was somewhat damaged by incubation in strong buffer, and also that removal of the buffer deprived the cells of some essential factor which, when added to other cells, stimulated their adaptation.

A further experiment was performed to investigate whether or not this factor was specific to tetrathionate reducing organisms, or connected with the metabolism of mannitol. The following mixtures were made up in duplicate:

	(i) (ml.)	(ii) (ml.)	(iii) (ml.)	(iv) (ml.)
Cell suspension of organism 1433	3	3	—	—
*Bact. coli** cell suspension	—	—	3	3
Buffer, M	3	3	3	3
Mannitol, 0·2 M	—	1·5	—	1·5
Water	6	6	6	6

* *Bact. coli* does not reduce tetrathionate.

These mixtures were incubated at 37° for 60 min., centrifuged, the cells discarded and the supernatant fluids added to: (A) four lots of packed cells of organism 1433 (from 3 ml. of stock suspension) which had been pre-treated with mannitol and buffer for 60 min.; (B) four lots of packed cells each spun off from 3 ml. of fresh stock suspension.

The cells were suspended in these fluids and after warming to 37° the reaction mixtures were completed by the addition of 1·5 ml. 0·2 M tetrathionate to all tubes and 1·5 ml. 0·2 M mannitol to tubes derived from (i) and (iii). Fig. 8, curve *a* shows a standard (untreated) adaptation and curve *b* the adaptation of cells after pre-treatment. Curves *d* show adaptations of pre-treated cells centrifuged off and resuspended in fluids (i)–(iv) as compared with the same cells in a new reaction mixture (curve *c*). Curves *e* show the effect of these fluids on the adaptation of fresh cells as compared with the normal adaptation (curve *a*). From these results it can be seen that the activity lost by pre-treated cells when separated from their suspending fluid was restored, more

or less equally, by fluids from reducer or non-reducer cells, with or without mannitol.

The suspending fluids each stimulated the adaptation of fresh cells to a roughly equal extent, but even so, these cells did not adapt so rapidly as the pre-treated cells, suggesting that the chief effect of pre-treatment is to build up intermediates inside the cells.

The cells. The cells themselves were then investigated. It was possible to extract a stimulatory factor from pre-treated cells by centrifuging them after treatment, suspending the deposit in a small volume of water and then placing the suspension in a boiling water-bath for 10 min. The suspension was then cooled, centrifuged and the active extract pipetted off. Some stimulation was given by extracts prepared in the same way from untreated cells and from cells stood in water (at room temperature or at 37°) and from cells treated at 37° in buffer only, but the extract from cells treated with mannitol and phosphate showed greater stimulation. Some of the stimulation of adaptation produced by these extracts might be due to assimilable nitrogen compounds which they contained, but the difference between extracts from untreated and pre-treated cells could only be due to substances produced during pre-treatment.

The stimulatory factor withstood heating at 100° at pH 7·3 for 60 min. When the extract was mixed with four times its own volume of 95% (v/v) ethanol a flocculent precipitate appeared (presumably polysaccharide material) and when this was centrifuged off all the activity was found in the ethanol-soluble fraction; the precipitate had no stimulatory effect. When the pH of the extract was brought to 3·5 a similar precipitate appeared. Again the precipitate was without stimulatory effect, all the activity remaining in the acid-soluble portion. Almost all the active part of the extract passed through a cellophan membrane in 4–5 days when the extract was dialysed against water at 4°. The material inside the sac (which included the presumed polysaccharide substance) was without stimulatory effect. The nitrogen content of the active fraction which passed through cellophan was 0·06 mg. N/ml. as compared with 3·8 mg. N/ml. in tryptic heart broth; it seems unlikely therefore that the stimulatory effect was solely due to assimilable nitrogen. The active principle has not so far been identified.

The effect of inhibitors

2:4-Dinitrophenol at 0·0005 M completely inhibited the adaptation of untreated cells (Fig. 10, curve *c*), but when added with the tetrathionate to pre-treated cells it did not prevent a slow adaptation (Fig. 10, curve *b*), suggesting that during pre-treatment there accumulated an intermediate produced at some point subsequent to the dinitrophenol block. Like dinitrophenol, sodium azide at 0·025 M inhibited adaptation, but not reduction of tetrathionate by adapted cells. However, unlike dinitrophenol, azide completely inhibited the adaptation of pre-treated cells (Table 3).

From these investigations it seems probable that most of the stimulation of adaptation which occurs when cells are pre-treated in mannitol and phosphate is due to intermediates of mannitol metabolism which accumulate within the

cells, though material essential for adaptation, and which apparently diffused out of the cells and was independent of the presence of mannitol, did appear in the suspending fluid.

Table 3. *Effect of inhibitors on the adaptation of pre-treated cells of organism* 1433

The following mixtures were made up and incubated:

	Controls Tetrathionate and inhibitor added at start		*Pre-treated cells* Tetrathionate and inhibitor added after 90 min.	
	(ml.)	(ml.)	(ml.)	(ml.)
Stock cell suspension	2	2	2	2
Buffer, M	2	2	2	2
Mannitol, 0·2 M	1	1	1	1
Sodium tetrathionate, 0·2 M	1	1	1	1
Sodium azide, 0·25 M	1	—	1	—
2:4-Dinitrophenol, 0·01 M	—	0·5	—	0·5
Water	3	3·5	3	3·5
	155 min. after the addition of tetrathionate the tetrathionase activity was measured by the method described			
Activity (μmol. thiosulphate produced/mg. dry wt./hr.)	0·2	0·2	0·2	1·7

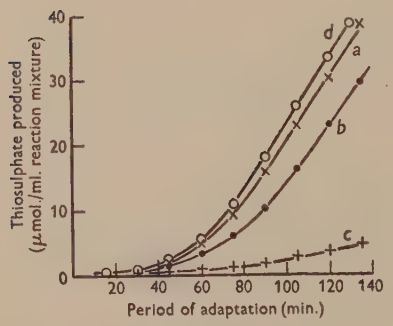


Fig. 9

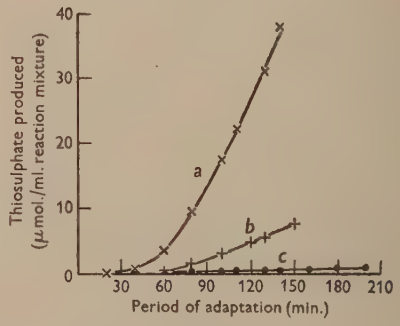


Fig. 10

Fig. 9. Effect of incubation of organism 1433 in 0·2 M-phosphate buffer (pH 7·6). Curve *a* (×——×), standard adaptation curve for comparison cells not previously incubated in buffer; curve *b* (●——●), cells incubated in buffer for 60 min., then rest of standard adaptation mixture added; curve *c* (+——+), cells incubated in buffer for 60 min., centrifuged off from supernatant fluid, and then added to new reaction mixture; curve *d* (○——○), supernatant fluid centrifuged off from cells incubated in buffer for 60 min., added to fresh cells and standard adaptation mixture completed.

Fig. 10. Effect of 2:4-dinitrophenol on the adaptation of untreated cells of organism 1433 and on cells pre-treated with mannitol and phosphate. Curve *a* (×——×), adaptation curve without dinitrophenol for comparison; curve *b* (+——+), dinitrophenol and sodium tetrathionate added after 90 min. incubation of cells with mannitol and phosphate; curve *c* (●——●), dinitrophenol and sodium tetrathionate added to untreated cells.

DISCUSSION

The results obtained are mostly an amplification of previous work on tetrathionase and a confirmation for this system of conclusions reached with other adaptive enzymes. There are, however, several points worthy of comment. It was observed that the rates of reduction in the presence of dinitrophenol and mannitol, lactate, formate or ethanol, respectively, are in descending order of magnitude (Fig. 3), not only when the cells are fully adapted, but throughout the adaptation, and an unpublished experiment showed that even the small activity of unadapted cells varies with the hydrogen donator used. These observations provide an unexpected example of the dangers (elaborated by Burton, 1936) of thinking in terms of a 'master reaction'—the idea that the overall rate of a process is determined solely by the slowest reaction of the chain. The different rates with different H donators were first observed with fully adapted cells and it was thought that unadapted cells, which show an overall velocity some forty to fifty times smaller, might reduce tetrathionate at the same rate with all H donators. But this is not the case. Tetrathionate reduction almost certainly consists of a chain of reactions catalysed by several enzymes, beginning with a dehydrogenase and terminated by an enzyme activating tetrathionate which is the adaptive tetrathionase enzyme. Even when this last is least active and might be expected to be solely responsible for the observed rate, the velocity of reduction is influenced by the specific H donator used, despite the fact that the slowest of these is not required to supply hydrogen at anything like its maximum rate. Reasonable doubts may be entertained as to the validity of using over-all rates of reduction as a measure of the content of adaptive enzyme, but until cell-free preparations can be obtained there is no alternative.

Monod (1947) observes that most adaptations have the substrate of the adaptive enzyme as the sole metabolite and, therefore, as the only source of energy, so that they might be expected to be in some way autocatalytic, as in fact the course of the adaptation does suggest. Tetrathionase adaptation will occur slowly in washed suspension with lactate as H donator and also when cells grow anaerobically in tetrathionate lactate medium (Knox *et al.* 1943). The fact that adaptation occurs so much more rapidly in the presence of a fermentable H donator would appear to indicate that energy from fermentation can be used for adaptation. The acceleration of adaptation produced by allowing fermentation to begin before adding the tetrathionate is in agreement with this. Further, it seems that in the early stages of adaptation this fermentation energy is either greater in amount, or more available to the adaptive process, than any energy from tetrathionate reduction. The rate of enzyme production does increase with time, for about 60 min., but there is no evidence to show whether or not this is due to more energy becoming available as the rate of tetrathionate reduction increases. It is tempting to suppose that as the rate of reduction increases the metabolism of the carbohydrate becomes oxidative rather than fermentative, with an increase in the available energy.

Adaptation does not occur in the presence of a free supply of oxygen.

Pollock & Knox (1943) showed that a free supply of oxygen inhibited tetrathionate reduction by fully adapted cells—this finding is analogous to Stickland's (1931) finding with nitrate—and as it has been shown that oxygenation destroys the tetrathionase of adapted cells it is not surprising that adaptation does not occur in the presence of a free oxygen supply. That the ability to produce new enzyme is not abolished by oxygenation is shown by the fact that adapted cells which have had their tetrathionase destroyed by oxygenation will still adapt, in much the same way as fresh cells, when they are transferred to a suitable environment. Since attempts to re-activate de-adapted cells by incubation with reducing agents were unsuccessful and since the presence of tetrathionate during oxygenation did have some protective effect, it seems possible that the loss of activity is due not to oxidation of some part of the system but rather to reversal of the adaptation process. This de-adaptation produced by exposing the cells to oxygen, the activating enzyme for which is presumably constitutive, appears analogous to the loss of adaptive enzyme attacking certain carbohydrates which occurs when yeast cells are allowed to ferment glucose (Dienert, 1900; Stephenson & Yudkin, 1936).

To review the evidence for believing that during adaptation an enzyme-activating tetrathionate is synthesized the following may be said: (*a*) Adaptation occurs without cell division and is not, therefore, a selection of variants. (*b*) Adaptation is not an increase in the dehydrogenating power of the cells (Pollock, 1946). During adaptation the ability of the cells to ferment mannitol and to take up oxygen in its presence is unchanged, or declines slightly (unpublished observations). (*c*) Adapted cells, but not unadapted, will activate tetrathionate to oxidize reduced Nile blue (Jebb, 1949). (*d*) Adaptation is stimulated by the presence of assimilable nitrogen, suggesting a synthesis of nitrogenous material, e.g. protein. (*e*) Rapid adaptation occurs only in the presence of fermentable carbohydrate, suggesting the need for energy in an anabolic biosynthetic process. (*f*) 2:4-Dinitrophenol, which stimulates reduction by adapted cells but inhibits adaptation, is known to be an inhibitor of synthetic processes (Reiner, 1946).

Although by no means conclusive, all this evidence is consistent with the view that new enzyme is synthesized in the adaptive process.

REFERENCES

Burton, A. C. (1936). The basis of the principle of the master reaction in biology. *J. cell. comp. Physiol.* **9**, 1.

Clifton, C. E. (1946). Microbial assimilations. *Adv. in Enzym.* **6**, 269.

Dienert, F. (1900). Sur la fermentation du galactose et sur l'accoutumance des levures a ce sucre. *Ann. Inst. Pasteur*, **14**, 139.

Gale, E. F. (1943). Factors influencing the enzymic activities of bacteria. *Bact. Rev.* **7**, 139.

Gilman, A., Phillips, F. S., Koelle, E. S., Allen, R. P. & St John, E. (1946). The metabolic reduction and nephrotoxic action of tetrathionate in relation to a possible interaction with sulphydryl compounds. *Amer. J. Physiol.* **147**, 115.

Gladstone, G. P. & Fildes, P. (1940). A simple culture medium for general use without meat extract or peptone. *Brit. J. exp. Path.* **21**, 161.

Jebb. W. H. H. (1949). The use of Nile blue in the study of tetrathionase activity. *J. gen. Microbiol.* **3**, 112.

Knox, R., Gell, P. G. H. & Pollock, M. R. (1943). The selective action of tetrathionate in bacteriological media. *J. Hyg., Camb.*, **43**, 147.

Knox, R. & Pollock, M. R. (1944). Bacterial tetrathionase: adaptation without demonstrable cell growth. *Biochem. J.* **38**, 299.

Kosterlitz, H. W. (1943). The fermentation of galactose and galactose-1-phosphate. *Biochem. J.* **37**, 322.

Monod, J. (1942). *Recherches sur la croissance des cultures bactériennes*, p. 117. Paris: Herman et Cie.

Monod, J. (1947). The phenomenon of enzymatic adaptation. *Growth*, **11**, 223.

Pollock, M. R. (1945). The influence of temperature on the adaptation of 'tetrathionase' in washed suspensions of *Bact. paratyphosum* B. *Brit. J. exp. Path.* **26**, 410.

Pollock, M. R. (1946). Adaptation of 'nitratase' in washed suspensions of bacteria. *Brit. J. exp. Path.* **27**, 419.

Pollock, M. R. & Knox, R. (1943). Bacterial reduction of tetrathionate. *Biochem. J.* **37**, 476.

Pollock, M. R. & Wainwright, S. D. (1948). The relationship between nitratase and tetrathionase adaptation and cell growth. *Brit. J. exp. Path.* **29**, 223.

Reiner, J. M. (1946). The effect of enzyme inhibitors on transformation of enzymes in the living cell. *Proc. Soc. exp. Biol., N.Y.*, **63**, 81.

Ronzoni, E. & Ehrenfest, E. (1936). The effect of dinitro-phenol in the metabolism of frog muscle. *J. biol. Chem.* **115**, 749.

Spiegelman, S. (1945). The physiology and genetic significance of enzymatic adaptation. *Ann. Mo. bot. Gdn*, **32**, 139.

Spiegelman, S., Reiner, J. M. & Morgan, I. (1947). The apo-enzymatic nature of adaptation of galactose fermentation. *Arch. Biochem.* **13**, 113.

Stephenson, M. & Yudkin, J. (1936). Galactozymase considered as an adaptive enzyme. *Biochem. J.* **30**, 506.

Stickland, L. H. (1931). The reduction of nitrates by *Bact. coli*. *Biochem. J.* **25**, 1543.

Wilkinson, J. F. (1949). The pathway of adaptive fermentation of galactose by yeast. *Biochem. J.* **44**, 460.

(*Received* 5 *December* 1949)

Tetrathionase: The Differential Effect of Temperature on Growth and Adaptation

By R. KNOX

Public Health Laboratory, University of Oxford

SUMMARY: Cells of a tetrathionate-reducing coliform organism growing in semi-anaerobic conditions gained no advantage from the presence of tetrathionate at 41°, but at lower temperatures (e.g. 34°) they grew much better with than without it. When freely supplied with oxygen, the cells grew about as well at 41° as at 34°. The adaptive formation of tetrathionase in washed suspension has already been shown to diminish with increase of temperature from 34° to 44°, whereas the activity of the enzyme when formed increases with temperature in this range; nitratase, on the other hand, is still actively formed at temperatures as high as 44°. It is clear that whatever factors may be necessary for adaptation, one of them is more sensitive to heat than either nitratase formation or the overall growth process.

In previous work the following facts about the bacterial enzyme system tetrathionase were established:

(1) Salmonellae and a number of other Gram-negative bacteria can reduce tetrathionate quantitatively to thiosulphate (Knox, Gell & Pollock, 1943). Both the activity of the fully developed enzyme and its adaptive formation can be demonstrated in washed suspension in the presence of a suitable H donator (Pollock & Knox, 1943; Knox & Pollock, 1944).

(2) The velocity of reduction by adapted cell suspensions increases with temperature at least up to 40° or even higher. Adaptation, on the other hand, is almost completely suppressed at 41–42°, while it occurs rapidly at 37° and even lower temperatures (Pollock, 1945).

(3) The reduction of tetrathionate is of value in growth to those organisms that can reduce it. Tetrathionate is a selective hydrogen acceptor and an alternative to oxygen; it is not so efficient as oxygen but it enables a broth culture to reach in a few hours a population two to three times the population reached in unaerated broth (Knox, 1945).

The relation between adaptation (which may involve the biosynthesis of a specific enzyme or enzyme system) and growth of a bacterial culture (synthesis of bacterial protoplasm as a whole) is one of intriguing interest. A study has now been made of the effect of temperature on the capacity of a bacterial culture to reduce tetrathionate during growth.

METHODS

The culture used was the coliform organism of intermediate type labelled '1433' (Pollock, 1946). Cells from an overnight broth culture prepared from stock agar slopes were heavily inoculated, usually so as to give just visible turbidity at the start, into 60–80% tryptic heart broth containing 0·05M phosphate at pH 7·6. Tetrathionate was used in a concentration of 0·02M. Cultures were incubated in thermostatically controlled water-baths at different temperatures,

static cultures in 6 in. × $\frac{3}{4}$ in. or 6 in. × $\frac{5}{8}$ in. tubes, but aerated cultures in larger tubes through which oxygen was bubbled and to which a few drops of undecanol were added to suppress frothing (Linggood & Fenton, 1947). Either the culture tubes themselves or suitable samples, diluted if necessary, were removed for estimation of turbidity in a Hilger Biochem absorptiometer. In some experiments total counts were performed using a Helber counting-chamber and dark-ground illumination, and viable counts by surface plating of suitable dilutions on to nutrient agar.

RESULTS

Fig. 1 illustrates the growth of organism 1433 in buffered broth at 34° and 41° under different conditions. In freely oxygenated broth growth at 41° was at least as good as at 34°. There was some variation in different experiments as it

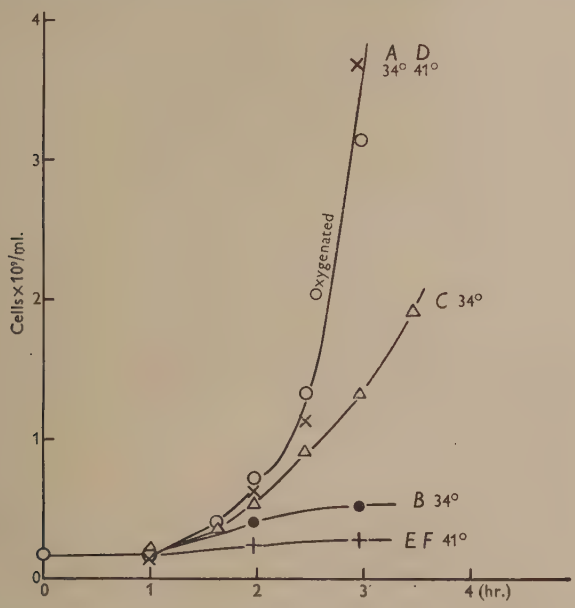


Fig. 1. Growth of organism 1433 in buffered broth at 34° and 41°. At 34°: *A*, oxygenated; *B*, semi-anaerobic; *C*, tetrathionate (0·02 M). At 41°: *D*, oxygenated; *E*, semi-anaerobic; *F*, tetrathionate (0·02 M).

was difficult to maintain exactly equivalent oxygenation at the two temperatures. In some experiments growth at 41° was initially a good deal faster than at 34°. In static broth cultures growth was poor at both temperatures as the limiting population was reached after only a few cell divisions, but growth at 41° was slightly less than at 34°. In tetrathionate broth growth at 34° was good, though the cultures never reached the same density as in oxygen, but at 41° it was poor. These experiments showed that although with a free supply of O_2 the cells grew equally well at 41° and at 34°, it was only at the lower

temperature that they gained an advantage from using tetrathionate as an alternative H acceptor to oxygen.

Measurement of tetrathionate reduction by the cells in this experiment at the two temperatures showed that at 34° tetrathionate was rapidly reduced to thiosulphate, whereas at 41° the amount reduced was negligible.

Fig. 2 shows for comparison the behaviour of adapted and unadapted cells of organism 1433 in washed suspension at the two temperatures used in the growth experiments. Reduction of tetrathionate at 41° was, as expected, faster than at 34°, whereas adaptation by unadapted cells was much faster at 34° than at 41°, although not completely suppressed at the higher temperature.

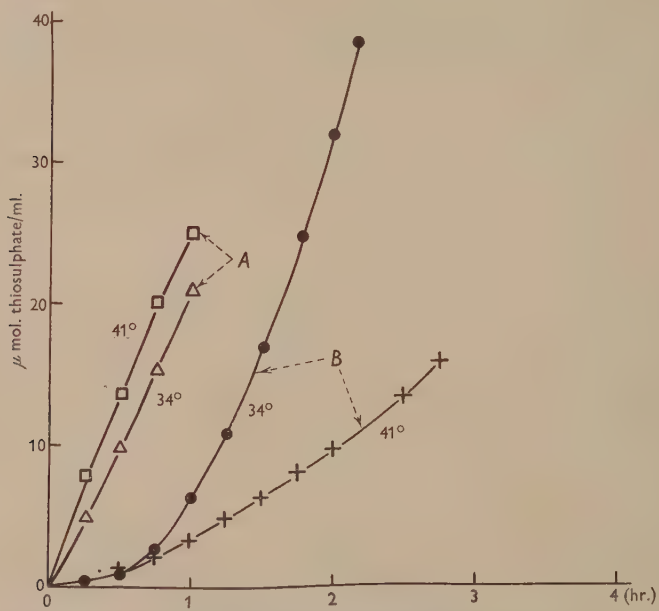


Fig. 2. Reduction of tetrathionate by washed suspensions of organism 1433 at 34° and 41°. *A*, adapted cells; *B*, unadapted cells.

It is not certain that adaptation during growth is the same process as adaptation in washed suspension, but it is interesting to observe that the known temperature-sensitivity of the adaptive mechanism in washed suspension is paralleled by the behaviour of the same organism during what may be described as 'adaptive' growth. It seems that adaptation is a necessary preliminary for adequate growth in semi-anaerobic conditions—since adequate growth occurs, even in a rich medium, only at temperatures low enough to leave undamaged the mechanisms of adaptation. There was found to be a fairly wide temperature optimum for 'adaptive' growth, between 32° and 38°. Above this range 'adaptive' growth still occurred at 40° but declined very sharply between 40° and 41°, until at 42° it was completely suppressed.

In view of the known selective value of tetrathionate, a few experiments were performed with mixed cultures of the tetrathionate reducer 1433 and a strain of *Bact. coli* which did not reduce tetrathionate. Tubes of heart broth and of tetrathionate heart broth, containing mixtures of the two organisms in about equal numbers, were incubated at 34° and 41°. The heart-broth tubes were freely oxygenated throughout the experiments. The results, as might be expected, were erratic; but in one experiment whereas the proportion of reducers to non-reducers either remained unchanged or decreased in oxygen at 34° and 41° and in tetrathionate at 41°, it increased in tetrathionate at 34°, showing that the reducer gained an advantage over the non-reducer in the presence of tetrathionate only when growing at a temperature at which adaptation and therefore utilization of the tetrathionate occurred.

The failure of organism 1433 to reduce tetrathionate during growth at 41° was presumably due to a noxious effect of temperature, and perhaps of tetrathionate as well, on some metabolic process in the cells. The least sensitive mechanism is almost certainly not the tetrathionase enzyme system, since in washed suspension this is more active at 41° than at 34°. Nor is it likely that the growth of the whole bacterial cells is much affected, since freely aerated cultures of organism 1433 grow as well at 41° as at 34°, not only in the absence, but also in the presence of tetrathionate. All the evidence suggests that it is the process of adaptation as opposed to the overall growth process that is differentially and selectively interfered with by the increase of temperature.

To what extent this inhibition of adaptation can itself be explained by an increased toxic effect of tetrathionate at higher temperatures is not clear. At this point some experiments with nitratase are relevant.

Cultures of organism 1433 were incubated in the usual way at different temperatures, but with nitrate instead of tetrathionate. At 41°, whereas growth in tetrathionate broth was very poor, in nitrate broth it was as good as at 34°, and nitrate still stimulated growth up to 42–43°. Even the presence of tetrathionate in addition only partially suppressed this stimulatory effect. These experiments suggested that nitratase adaptation is less thermolabile than tetrathionase adaptation. This has been further demonstrated by direct experiments on the effect of temperature on the two adaptive processes in washed suspension. A considerable adaptive production of nitratase occurred even at 44·5°, whereas tetrathionase adaptation was almost abolished at 42° and considerably inhibited at 40° (Pollock & Wainwright, unpublished observations). Elsewhere we have shown (Jebb, Knox & Tomlinson, 1950) that tetrathionate concentrations down to 0·005 M do have some inhibitory effect on adaptation; but since the effect of tetrathionate on 'adaptive' growth in nitrate is not great even at 42° it seems unlikely that tetrathionate should suppress the formation of 'its own' enzyme even more than that of another. Finally, it was found in growth experiments in the presence of tetrathionate at 34° and 42°, that 'adaptive' growth still failed to occur at the higher temperature even when the tubes contained as little as 0·0025 M tetrathionate.

DISCUSSION

If tetrathionate is to give an effective stimulus to the growth of cells which can use it as a hydrogen acceptor when oxygen is limited, it is clear that growth must occur at a temperature at which adaptation can take place, namely, at around 32–37°. At higher temperatures, appreciable growth in a medium in which tetrathionate is the only effective hydrogen acceptor fails because adaptation is suppressed. This suppression may in turn be merely a reflexion of the fact that tetrathionate is a somewhat toxic substance whose toxicity might be expected to increase with rise in temperature. But whatever the explanation, it is evident that here is an example of a specific adaptation process being almost completely suppressed at a temperature at which growth seems quite unimpaired. The reverse might perhaps have been expected, since increase in bacterial protoplasm (growth) is presumably the result of the co-ordinated working of many enzyme systems, and the optimum temperature for growth no doubt depends on a balance resulting from the temperature optima of many of these systems, the most heat-sensitive of which might be expected to be growth-limiting. With tetrathionase it seems that while this enzyme system when formed is less sensitive, the actual process of its formation is much more sensitive to increase in temperature than is the growth process as a whole. There seems to be little information as to the heat-sensitivity of other adaptive enzymes. It is interesting that nitratase adaptation seems to be much less heat-sensitive than tetrathionase; this is further evidence of the comparative independence of these two adaptation processes.

It is only in recent years that the importance of adaptation in bacterial growth has been appreciated, and an investigation into the temperature optima of different adaptive systems may be expected to add considerably to our understanding of the growth processes of the bacterial cell.

My thanks are due to Dr M. R. Pollock and Dr S. D. Wainwright for the experimental data referred to in the text.

REFERENCES

Jebb, W. H. H., Knox, R. & Tomlinson, A. H. (1950). Some factors influencing the rate of formation of tetrathionase. *J. gen. Microbiol.* **4**, 374.

Knox, R. (1945). The effect of tetrathionate on bacterial growth. *Brit. J. exp. Path.* **26**, 146.

Knox, R., Gell, P. G. H. & Pollock, M. R. (1943). The selective action of tetrathionate in bacteriological media. *J. Hyg., Camb.*, **43**, 147.

Knox, R. & Pollock, M. R. (1944). Bacterial tetrathionase: Adaptation without demonstrable cell growth. *Biochem. J.* **38**, 299.

Linggood, F. V. & Fenton, E. L. (1947). The production of diphtheria toxin by submerged culture in shaking flasks. *Brit. J. exp. Path.* **28**, 354.

Pollock, M. R. (1945). The influence of temperature on the adaptation of 'tetrathionase' in washed suspensions of *Bact. paratyphosum* B. *Brit. J. exp. Path.* **26**, 410.

Pollock, M. R. (1946). Adaptation of 'nitratase' in washed suspensions of bacteria. *Brit. J. exp. Path.* **27**, 419.

Pollock, M. R. & Knox, R. (1943). Bacterial reduction of tetrathionate. *Biochem. J.* **37**, 476.

(*Received* 5 *December* 1949)

Simplified Fluorescence Microscopy of Tubercle Bacilli

By E. MATTHAEI

University of Melbourne, Australia

SUMMARY: The fluorescence of tubercle bacilli stained with auramine and rhodamine does not require ultra-violet light. It can be caused by blue light up to 496 mμ. A copper sulphate ammonia liquid filter, suitably diluted, will transmit this wave-band. A gelatin screen filter, absorbing all light below 510 mμ. is used in the eyepiece. Normal, high intensity projection filament lamps, combined with lamp condensers of large aperture, provide suitable light sources. The numerical aperture of the microscope condenser must be fully used by immersion in glycerol. The fluorescence is very bright with the usual biological, and certain binocular, microscopes. The simplicity of the equipment enables fluorescence microscopy of tubercle bacilli to be used at low cost in any laboratory.

The phenomenon of fluorescence as applied to stained bacilli may be described as follows. Certain fluorochroms (e.g. auramine) absorb light of a certain wave-length, and convert it into light energy of another wave-length. This effect is observed by illuminating the specimen with suitable light passed through a filter which transmits a wave-band capable of causing fluorescence, and by incorporating into the observing part of the microscope a filter which transmits the colour of the fluorescence, but absorbs the light which has caused it.

The use of auramine as an acid-fast stain for the examination of tubercle bacilli in fluorescent light under the microscope was first introduced by Hagemann in 1938. Later workers made several improvements in Hagemann's method, but these were mainly technical. In essence the principle has remained the same, and is to-day recognized as having great advantages over the usual Ziehl-Neelsen technique: the staining process is quick and the result very clear-cut because of the great contrast between bacilli and background, the appearance of the field being almost that of a dark-ground with the bacilli a bright golden-yellow colour. Moreover, because of the distinctive appearance of the bacilli, a much lower magnification than usual can be used: $\times$ 300–400 instead of the usual $\times$ 1350. Thus an area from ten to twenty times that of the average oil-immersion field can be covered, and the time of searching the slide correspondingly decreased.

The instruments used in the early development of fluorescence microscopy were equipped with a quartz optical system and a carbon arc with ultra-violet filter to produce the necessary fluorescence. Later workers (see Ellinger, 1940) considered that the fluorescence could be obtained with light of 300–400 mμ. wave-length, and since most of this is transmitted by the usual optical glass, the use of special quartz lenses and condensers became unnecessary. The mercury arc, with a very strong emission band at 365 mμ., is now used as the most practicable light source, together with a liquid copper sulphate ammonia filter to absorb all the light of more than 436 mμ.

Richards, Kline & Leach (1941) described a high-intensity low-voltage

filament lamp with high ultra-violet emission, together with a blue ultra-violet transmitting glass filter, which again simplified the equipment necessary to produce fluorescence,

Earlier, Keller (1938) had achieved fluorescence of tubercle bacilli by means of a glass filter with a transmission maximum in the violet, using a 6 V. 5 amp. filament lamp as source of light. This arrangement, though simple, lacked intensity. Strugger (1939) described it as 'sehr lichtschwach'.

The purpose of the present paper is to show that adequate fluorescence of auramine can be produced by light up to 495 mμ. The whole of the blue part of the spectrum is thus used. With such a wide wave-band, most high-intensity projection-type filament lamps (readily obtainable everywhere) give sufficient intensity to cause good fluorescence.

Some confusion seems to exist about the photometry of fluorescence microscopes. I therefore investigated the problem of how best to utilize light sources and lamp condensers in combination with the numerical aperture of the microscope and its condenser.

EXPERIMENTAL

The kind of light needed for fluorescence was determined by first using a mercury-vapour lamp with a Wratten filter No. 50 and a 4 cm. layer of 2 % aqueous copper sulphate. This filter is practically monochromatic and transmits the blue violet light of 435/6 mμ. and a small amount of the violet 408 mμ. The tubercle smears were stained (see below) with auramine and rhodamine. A Wratten gelatin filter G15, absorbing all light below 510 mμ., was used as screen-filter in the eyepiece. With a magnification of $\times$400, well-defined tubercle bacilli were seen against an almost black background.

Since, therefore, the 435/6 mμ. mercury lines are capable of producing the required fluorescence, a copper sulphate ammonia liquid filter was substituted for the Wratten filter No. 50. This liquid filter is described by Bowen (1942) in combination with a sodium nitrite filter for the isolation of the 435/6 mμ. lines. If used without the sodium nitrite filter and altered in concentration, it can be made to transmit the whole of the blue spectrum including the 492/6 mμ. lines. Tests in the Beckman spectrophotometer showed that the transmission goes down as far as 365 mμ.

The best working concentration of the filter was arrived at by diluting the copper sulphate ammonia solution until the 492/6 mμ. lines were just visible when seen through a hand spectroscope against a mercury-vapour arc. It is not claimed that the 492/6 mμ. lines themselves contribute to the fluorescence. But they certainly do not interfere with it, and make it possible to obtain greater intensities by using a transmission band up to 500 mμ. Since it is unnecessary to use a mercury lamp to produce light above 400 mμ., a normal microscope lamp, as used for critical illumination, was substituted. Its lamp was of the condensed-filament projection type (12 V., 100 W.) in a suitable lamp-house with a 2 in. biconvex condenser. Results obtained with this lamp were very good.

The energy distribution of the projection filament lamps was ascertained.

They had an average colour temperature of 2900° K. (Pl. 1, fig. 1). Their radiant energy at 365 mμ. is only some 5%, rising to 9% at 400 mμ. and to *c.* 28 % at 500. The actual energy contribution in the ultra-violet region below 400 mμ. is therefore very small. Even a 500 W. projection filament lamp, used with an ultra-violet filter to isolate the 365 mμ. line, will not produce a noticeable fluorescence. It can be concluded that a normal projection filament lamp of sufficiently high intensity, used at its normal colour temperature and at its proper voltage, is capable of producing a good fluorescence with auramine-stained tubercle bacilli, provided the transmission band of the filter is wide enough to let through enough blue light.

The apparatus and its use

The problem was investigated with a number of different microscopes, monocular and binocular, and with all readily available projection filament lamps used in a number of different lamp-housings. The following standard equipment is satisfactory:

Microscope. Monocular with a normal 4 mm. objective, N.A. *c.* 0·65, primary magnification *c.* ×40, eyepiece ×7 or ×10 with a G15 Kodak Wratten gelatin filter cut to size and placed over the field lens inside the eyepiece. (This position is better than that of the field stop, where irritating blemishes are clearly visible.) Condenser: normal Abbe two lens type, with N.A. 1·2. Filament lamp: 12 V., 100 W.

Lamp-housing (Pl. 1, fig. 2). Tubular with biconvex condenser lens $f = 50$ mm., diameter = 50 mm.

Filter (*A*) (Pl. 1, fig. 3). The tube is made of phenolic resin, 7·6 cm. outside diameter, 6·3 cm. inside diameter, 5 cm. long; two rubber rings form a liquid-tight joint with the glass plates 3 mm. thick; two bakelite plates with appropriate apertures hold the filter together with four metal rods.

Filter (*B*) (Pl. 1, fig. 4). Alternative to (*A*), above, an 8 oz. white glass, plane-walled medicine bottle, outside measurements $1\frac{3}{4} \times 2\frac{1}{2} \times 7$ in., inside clearance *c.* 1 in. Such a bottle is adequate as a filter, though not as good as (*A*). The liquid in the filters has the following compositions: $CuSO_4.5H_2O$, 8·33 g.; conc. aqueous ammonia (sp.gr. 0·880), 100 ml.; water (for 5 cm. layer thickness filter *A*), 333 ml.; or (for 2·5 cm. layer thickness, filter *B*), 166 ml.

These filter concentrations are given as a guide. In individual cases they depend on such factors as the colour temperature and blue emission of the lamp. The best gauge of the filter concentration is the appearance of the stained smear. It should be such that golden-yellow tubercle bacilli are seen against a drab olive-green background. Dilution of the copper sulphate ammonia solution with water increases the width of the transmission band of the filter and the intensity of the field until the end of the blue part of the spectrum is reached. Further dilution quite suddenly changes the appearance of the background from a dark olive-green to a luminous blue-green, against which the fluorescent bacilli are difficult to distinguish.

Condenser. In normal microscopy the iris diaphragm of the condenser is used to bring about a balance between the desired definition and the required

depth of focus. Therefore, except with oil-immersion objectives, the aperture of the condenser is never fully used. The system is entirely different in fluorescence microscopy. The fluorescing particles are self-luminous, and, independently of the condenser, they will fill the whole aperture of the objective. In consequence, no amount of 'stopping down' will increase the definition, nor influence the depth of focus. The condenser is in fact used to get as great an intensity of blue light as possible to the specimen. The average condenser has N.A. 1·2 if there is an immersion medium of a refractive index of not less than 1·2 between condenser and slide; when the condenser is used dry, the resulting N.A. cannot be more than 1. Since the light-gathering power of a condenser is proportional to the square of its N.A., an immersed condenser of N.A. 1·2 will transmit 44 % more light than when dry, and a condenser with N.A. 1·4 will produce 96 % more light. Cedarwood oil, because it has a fluorescence of its own, is not advisable as an immersion medium. Glycerol ($n_D = 1{\cdot}463$) is non-fluorescent and suitably viscous. The condenser should always be used with the iris diaphragm wide open. Canada balsam, still the usual cementing medium for optical surfaces, has, like cedarwood oil, a fluorescence of its own which increases with age. Complex condensers with many cemented surfaces are therefore to be avoided.

The same considerations apply to the photometry of the objectives as to that of the condensers. Taking a final magnification of ×400 as a basis, a 4 mm. objective ×40, N.A. 0·65, with an eyepiece ×10 will yield *c.* 2½ times the amount of light as will an 8 mm. objective ×20, N.A. 0·40, with a ×20 eyepiece. These results were confirmed by measurements with a photoelectric cell. With dry objectives, a cover-glass which may be loosely placed over the smear, is normally required. Alternatively, objectives specially corrected for use without cover-glass are needed for uncovered specimens.

The lamp. As an optical instrument the lamp must fulfil the following conditions. An image of the lamp filament is projected into the plane of the microscope condenser diaphragm. It must be of a size to fill the whole of the back lens of the condenser. The standard lamp mentioned earlier has a bi-convex lens of 50 mm. diameter and 50 mm. focal length to produce this image. This corresponds to a numerical aperture of 0·45, and again conforms to the guiding rule to get as much light as possible into the microscope. A very efficient lamp can be constructed from a suitable tin container, with a spherical flask as a condenser (Pl. 1, fig. 4). With a 250 W. 115 V. tubular projection filament lamp, enough light is obtained even for a binocular microscope. But to achieve this result it is essential that the projection filament lamps be used at their proper voltage. A diminution in voltage, though it will prolong the life of the filament lamp, will decrease the colour temperature considerably and the blue part of the spectrum will suffer most of this loss.

Other microscopes. Monocular microscopes, at the same magnifications and N.A.'s of objective and condenser will produce fluorescent images of practically the same intensity. There are small contributory factors, such as bloomed lenses which transmit more light, and the use of an aluminized mirror instead of a back-silvered one. The general result is not greatly influenced by these.

Fluorite lenses, because of internal fluorescence, have not been found very satisfactory.

Binocular microscopes differ very considerably in their construction. The optical mechanism which splits the image into its two fields is made up of prisms. The number of prisms and the number of air/glass surfaces affect the light transmission, which differs widely in binocular microscopes of various makes. This loss of light is of no great importance in normal microscopy, but it is disastrous for the fluorescence technique when used with the intensities and light sources outlined in this paper. With binocular microscopes, the best results were obtained with the Zeiss L type (Pl. 1, fig. 2). With aplanatic condenser N.A. 1·4, lamp-house with spherical flask as condenser (Pl. 1, fig. 4), and a projection filament lamp of 250 W., 150 V. a good binocular image is obtained. The Watson Service binocular microscope, under otherwise the same conditions, produces only a faint image. Other makes of instruments, as far as they were available for test, fell in between the above two extremes. Though a dark room is not necessary for this system of fluorescence microscopy, a cover over lamp, filter-bottle and stage is advisable for protection from extraneous light.

Staining technique

Since Hagemann's use of auramine in 1938, a number of variations of his staining technique have been recommended, all aiming at an increase in intensity of the fluorescence. Hughes (1946) described a combination of auramine, rhodamine and acridine yellow which gives a very good orange-red fluorescence. The commercially obtainable auramine and rhodamine are satisfactory; the acridine yellow will give indifferent results unless carefully prepared. The Department of Bacteriology of the Melbourne University has for several years used the following combination of auramine and rhodamine with success. The stain is prepared by mixing Auramine OS (Imperial Chemical Industries Ltd.) 3 g., Rhodamine BS (Imperial Chemical Industries Ltd.) 1·5 g., and glycerol (Merck) 150 ml. Phenol crystals liquefied at 50° (20 ml.) are added, and then 100 ml. distilled water. The stain, heated to 50–60°, is poured on to smears fixed at a temperature of 70–75°, left for 10 min. and washed off with tap water. The preparation is decolorized with 0·5 % (v/v) conc. hydrochloric acid in 70 % (v/v) ethanol in water, using three changes, the first for 1 min., and the second and third for 2 min. each. The slide is dried on a hot plate at 52°.

Acknowledgement is due to the Department of Bacteriology of the Melbourne University for the preparation of the slides and description of the staining technique. I also wish to thank Assoc. Prof. W. A. Rawlinson for the evaluation of the blue filter transmission band.

REFERENCES

Bowen, E. J. (1942). *The chemical aspects of light.* Oxford: Clarenden Press.

Ellinger, P. (1940). Fluorescence microscopy in biology. *Biol. Rev.* **15**, 323.

Hagemann, K. H. (1938). Fluoreszenzfärbung von Tuberkelbakterien mit Auramin. *Münch. med. Wschr.* **85**, 1066.

Hughes, G. C. (1946). An improved stain for fluorescence microscopy. *Tubercle*, **91**.

Keller, Ch. J. (1938). Eine vereinfachte Methode zur Auffindung von Tuberkelbazillen im Fluoreszenzlicht. *Münch. med. Wschr.* **85**, 2024.

Richards, O. W., Kline, E. K. & Leach, R. E. (1941). Demonstration of tubercle bacilli by fluorescence microscopy. *Amer. Rev. Tuberc.* **44**, 255.

Strugger, S. (1939). Die Anwendung der Luminiszenzmikroskopie in der Botanik. *Zeiss Nachr.* Jena, **1**, 69.

EXPLANATION OF PLATE

Fig. 1. Energy distribution chart of high-intensity projection filament lamps showing proportion of ultra-violet and blue emission.

Fig. 2. Binocular microscope set up for fluorescence microscopy with filter vessel and microscope lamp normally used for critical illumination.

Fig. 3. Vessel for copper sulphate ammonia filter with phenolic resin spacer, glass covers and bakelite end-plates.

Fig. 4. Simple arrangement for observation of fluorescence with projection filament lamp in tin container, spherical flask as lamp condenser, and medicine bottle as filter vessel.

(*Received* 16 *December* 1949)

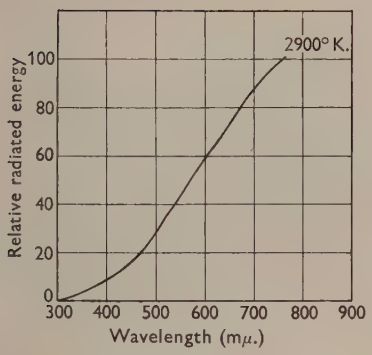


Fig. 1

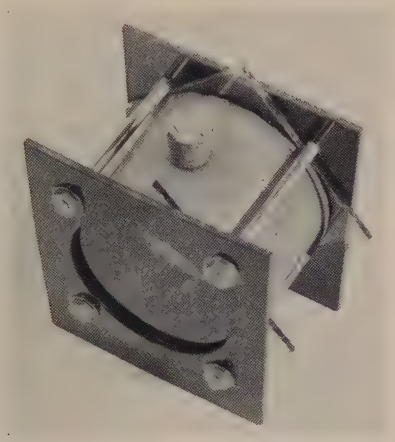


Fig. 3

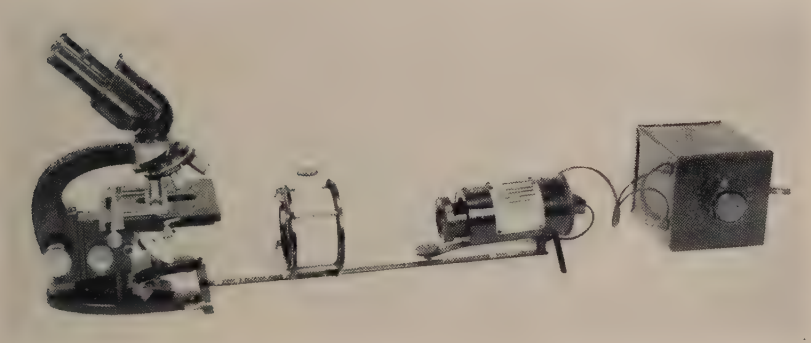


Fig. 2

Fig. 4

E. Matthaei—Simplified fluorescence microscopy of tubercle bacilli. Plate 1

Spectrophotometric Estimation of Nucleic Acid in Bacterial Suspensions

By P. MITCHELL

Department of Biochemistry, University of Cambridge

SUMMARY: A method is described for measuring the purine-pyrimidine absorption of *Micrococcus pyogenes* var. *aureus* (strain Duncan) (*M. pyogenes*; *Staphylococcus aureus*) in intact cell suspensions with the Beckman Model DU spectrophotometer, from which values for the total 'nucleic acid' content of the cells may be obtained. The approximate nature of the method is discussed, but results are presented which show that the values obtained throughout a normal growth of a culture of *M. pyogenes* compare very favourably with the values given by the Schmidt & Thannhauser (1945) technique. Some observations on *Escherichia coli* (strain H) suggest that the method may be applicable to organisms other than *Micrococcus pyogenes*.

In order to facilitate study of the effects of penicillin upon growing *M. pyogenes* (Mitchell, 1949*a*), a method of estimating total nucleic acid was sought which would be rapid and simple, applicable to small quantities of growing culture or washed suspension and capable of yielding relative values sufficiently accurate to show differences in the pattern of change of the nucleic acid content of normal and treated cells.

The available chemical methods based upon those of Schneider (1945) using pentose estimations, or Schmidt & Thannhauser (1945) using phosphorus estimations, required more than the amount of material initially available; the application of Caspersson's microspectrophotometric technique to organisms as small as staphylococci, although shown to be possible by Malmgren & Heden (1947), seemed too uncertain and too laborious to be suitable.

The uniformity of particle size and the known reproducibility of the scattering of white light by bacterial suspensions (Longsworth, 1936; Hershey, 1939) offered promise for the development of a method based upon direct spectrophotometric examination of intact cell suspensions. This paper describes an examination of the scattering and absorption spectra of *M. pyogenes* suspensions which forms the basis of a method of estimating total nucleic acid.

METHODS

Preparation of suspensions

M. pyogenes var. *aureus* (Duncan) was grown at 25° on a medium consisting of a tryptic digest of casein with 1·0% glucose and 0·1% Marmite. The rotated flask-culture method (Mitchell, 1949*b*) was employed to give standard and favourable growth conditions.

The reproducibility of the growth curves obtained showed that the physiological state of the cells was more accurately specified by the suspension density than by the age of the culture. The suspension density at harvesting is consequently taken as the index of the physiological condition of the cells used for the photometric studies.

Escherichia coli (H) was grown at 37° in 5 ml. lots of the casein digest medium in test tubes.

Washed suspensions of *Micrococcus pyogenes* were prepared by three rapid centrifugal washings with distilled water. *Escherichia coli* was washed similarly with three changes of a phosphate buffered saline of composition $Na^+:K^+:Mg^{++}=30:3:1$, $Na^+=0{\cdot}08$ M; $Cl':HPO_4':H_2PO_4''=6:1:1$, $Cl'=0{\cdot}06$ M; ionic strength 0·1.

Dry-weight determinations were made on washed *Micrococcus pyogenes* suspensions by drying a volume of suspension containing about 20 mg. of cells to constant weight at 105° in an air oven.

All photometric estimations were made in quartz cuvettes holding 3 ml. of suspension and giving a light path of 1 cm. Blank determinations were made with the cuvette filled with distilled water (saline for *Escherichia coli*), before every group of estimations on a new suspension.

Scattering spectrum

When the transmission of a dilute washed suspension of *Micrococcus pyogenes* (density at harvesting 2·2 mg./ml.) in distilled water was determined in the Beckman Model DU spectrophotometer for wave-lengths between 2000 and 350 mμ., the points of Fig. 1 were obtained. The smooth curve which fits the points well from 1000 to 350 mμ. was drawn by means of the simple inverse law,

$$\log_{10}(I_0/I)=\text{constant}.$$

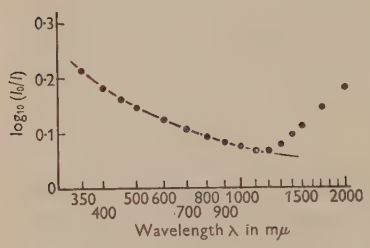


Fig. 1

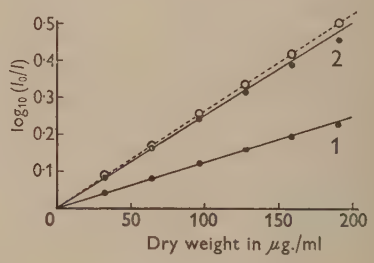


Fig. 2

Fig. 1. Scattering spectrum of *M. pyogenes* suspension of density 84·5 μg./ml. in water. Population density of culture at harvesting 2·2 mg./ml. Full curve, the inverse law.

Fig. 2. Relationship between dry weight of suspension and optical density due to scattering and absorption. Solid points and full lines: optical density due to scattering, 1 at 700 mμ., 2 at 350 mμ. Open circles and broken line: computed component of optical density at 260 mμ. due to absorption.

The optical density plotted against suspension density for wave-lengths of 700 and 350 mμ. gave the solid points of Fig. 2. It will be observed that for values of suspension density (m), up to 100 μg./ml., $\log_{10}(I_0/I)$ is proportional to m; so that the scattering spectrum between 1000 and 350 mμ. can be represented by the relationship

$$\log_{10}(I_0/I)=km/\lambda,$$

where k is 0·90 if λ is expressed in mμ. and m in μg./ml.

The constituents of the cells absorb very little light of wave-lengths between 2000 and 350 mμ., and we may consider the plot of the optical density against wave-length over this range to represent the scattering spectrum of the cells. It must, however, be borne in mind that transmission spectra due to scattering are dependent upon the aperture of the light-sensitive cell of the photometer, because of collection of part of the forward-scattered light with the transmitted part of the incident beam. As a result, unless special precautions are taken, scattering spectra are to some extent characteristic of the photometer used to measure them.

The minimum in the scattering spectrum of suspensions of *M. pyogenes* at 1200 mμ. and the sharp rise towards 2000 mμ. in the near infra-red were not expected, and it was at first thought that they might be due to light absorption. This possibility was eliminated when it was found that the scattering spectra of

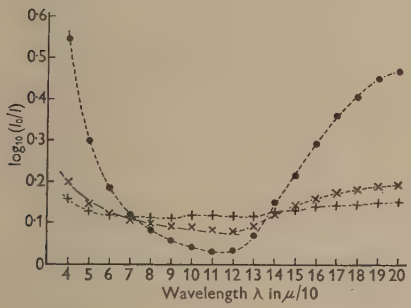


Fig. 3

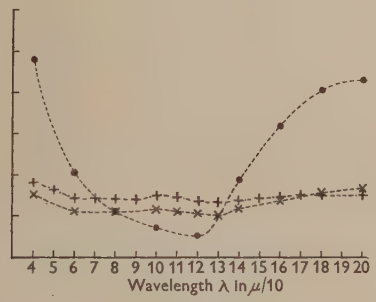


Fig. 4

Fig. 3. Scattering spectra of oleic acid particles in water. Solid points (- - ● - - ● - -), submicroscopic particles; oblique crosses (- - × - - × - -), particles 0·7–2 μ. in diameter; erect crosses (- - + - - + - -), particles 4 μ. in diameter; full line, the inverse law. Concentrations arbitrary.

Fig. 4. Scattering spectra of glass particles. Solid points (- - ● - - ● - -), particles of diameter $<0{\cdot}5$ μ. in water; erect (- - + - - + - -) and oblique (- - × - - × - -) crosses, particles of diameter 5 μ. in water and medicinal paraffin respectively. Concentrations arbitrary.

oleic acid particles of various sizes in water (Fig. 3) and glass particles in water and in medicinal paraffin (Fig. 4) were of the same general form. In all cases there was a minimum near 1200 mμ., but the rise in the scattering power of the suspension on either side of the minimum was steeper the smaller the particles. Only for the oleic acid suspension, which was found by microscopic examination to consist of particles between 0·7 and 2 μ. in diameter, did the scattering spectrum approximate closely to the inverse law (full curve). This suggested that the changes in cell diameter which occur during growth might be accompanied by changes in the scattering characteristics.

The scattering spectrum of a suspension of cells harvested in the early part of the log phase of growth (density at harvesting 0·3 mg./ml.) was found to approximate to the inverse law as closely as the suspension of Fig. 1, but the scattering coefficient k was 5 % lower than for the older cells. The value of

k at 350 mμ. (k_{350}) and the ratio of the values of k at 350 and 700 mμ. (k_{350}/k_{700}) taken at intervals throughout a normal growth are set out in Table 1. The figures indicated that in the late lag phase, the scattering spectra were flatter, while at the approach to the stationary phase they were steeper than the inverse law. This is in conformity with the observation that the cells were larger in the earlier phases of the growth of the culture. The theoretical value of the scattering power at 350 mμ., calculated from that at 700 mμ. by means of the inverse law, gives a deviation from the experimental value not greater than 11 %.

Table 1. *Slope of scattering spectrum of* Micrococcus pyogenes *during growth of culture*

Time (hr.)	Growth (μg./ml.)	k_{350}	k_{350}/k_{700}
0	51	0·93	0·89
2·5	72	0·97	0·89
5·5	214	0·94	0·94
7·5	475	0·94	0·99
10·5	1220	0·85	1·03
13·5	2480	0·84	1·08
16·5	3660	0·92	1·06
21·5	3800	0·97	1·05

$$k_\lambda = \left(\log_{10}\frac{I_0}{I}\right)_\lambda \times \frac{\lambda}{m}.$$

Absorption spectrum

On extending the spectrophotometric measurements into the ultra-violet, it was found that the cells not only scattered the incident light but also absorbed it. Fig. 5 shows that there are two disturbances, one at 260 mμ. and the other at about 280 mμ., corresponding to the ultra-violet absorption maxima of purines and pyrimidines and of aromatic amino-acids. In order to confirm that the deviations from the scattering spectrum indicated by extrapolation of the inverse law were due to absorption and not to an anomalous scattering phenomenon, the effect of changing the refractive index of the suspension medium was determined; for it would be expected that, other things being equal, a change of refractive index difference between the suspended particles and suspension medium should affect the scattering power of the suspension without altering its light absorption. The transmission spectra of the same batch of cells were determined in 40% aqueous and pure glycerol at the same suspension density as was employed for the measurements in water. The results plotted in Fig. 5 show that the scattering was reduced to about 1/5 in pure glycerol, while it had an intermediate value in 40 % glycerol. In all three cases the simple inverse law gives quite a good fit for the scattering spectrum between 1000 and 350 mμ. On the other hand, the increment of $\log_{10}(I_0/I)$ at 260 mμ. was nearly independent of the glycerol concentration (0·298 in water, 0·308 in 40% glycerol, 0·290 in absolute glycerol). It thus appeared justifiable to extrapolate the inverse scattering law from the visible region, where the cells do not absorb, to the near ultra-violet, where absorption did occur.

We now proceed by assuming that the Beer-Lambert law applies to the 'nucleic acid' in the cell suspension as if it were in homogeneous solution in the same volume, and that the absorption and scattering spectra contribute additively to the transmission spectrum. On this basis, the increment of $\log_{10}(I_0/I)$ at 260 mμ. above the value given by the inverse scattering spectrum

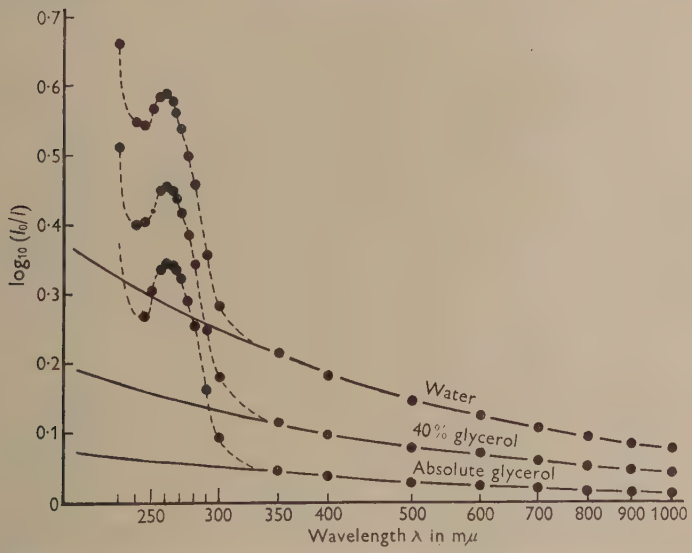


Fig. 5. Effect of refractive index of the medium upon the scattering + absorption spectrum of *M. pyogenes*. Suspension density 84·5 μg./ml. in all cases. Population density at harvesting 2·2 mg./ml. Full lines, the inverse law.

extrapolated from 350 mμ. may be taken to represent the 'nucleic acid' absorption. In other words, the percentage by weight of 'nucleic acid' in the cells can be arrived at by means of only two measurements, namely of $\log_{10}(I_0/I)$ at 350 and at 260 mμ. The dry weight is determined from $\log_{10}(I_0/I)$ at 350 mμ. and the value of k_{350}; the scattering contribution to the optical density at 260 mμ. is taken as 350/260 times $\log_{10}(I_0/I)$ at 350 mμ., and this value subtracted from the observed value of $\log_{10}(I_0/I)$ at 260 mμ. to give the absorption due to 'nucleic acid'. Accepting the value of 200 for the percentage decadic absorption coefficient ($=(1/cd)\log_{10}(I_0/I)$, where d = light path in cm. and c = concentration in % (w/v)) given by Malmgren & Heden (1947), the cells of Fig. 5 were found to contain 18 % by weight of 'nucleic acid'.

A plot of suspension density against apparent nucleic acid absorption (open circles of Fig. 2) gave a straight-line relationship over a broader range than held for the plot of optical density against suspension density. Consequently, when estimations are done on suspensions of greater density than 100 μg./ml., the percentage by weight of 'nucleic acid' comes out too high unless k is appropriately adjusted to correct for the non-linearity of the relationship between optical density and dry weight. The necessity for such corrections can

readily be avoided by using suspensions of densities between 30 and 100 μg./ml. for making the estimations.

While it is obvious that several of the assumptions which it has been necessary to make may be only partially justified, and the results consequently in error as absolute measurements by quite a wide margin, we should expect the comparative measurements to be less inaccurate. Fig. 6 shows duplicate experiments in which the percentage by weight of 'nucleic acid' (% NA) in *M. pyogenes* has been estimated throughout a normal growth period (open and closed circles). The complete cycle of % NA change is exhibited, and although

Table 2. *The evaluation of* % NA *during growth of* Micrococcus aureus *and comparison with phosphorus method of Schmidt & Thannhauser*

	Estimation of growth in the medium		Estimations on the washed suspensions											
			Optical density in mμ. at				Optical density 260 mμ.				By Schmidt & Thannhauser % wt.			
Time (hr.)	Optical density (700 mμ.) (i)	Dry wt. μg./ml. (700 mμ.) (ii)	700 (iii)	350 (iv)	260 (v)	Dry wt. μg./ml. (350 mμ.) (vi)	Scat. (vii)	Absn. (viii)	NA in μg./ml. (ix)	% NA (x)	Desoxyribo.		Total	Ratio S. & T. / U.V.
0	—	—	57	115	274	45	155	119	5·9	13·1	—	—	—	—
2	93	72	76	152	394	59	204	190	9·5	16·1	2·32	20·9	23·2	1·44
5	204	159	56	108	354	42	145	209	10·4	24·7	3·02	27·8	30·8	1·25
8	637	495	51	103	325	40	139	186	9·3	23·2	3·00	28·0	31·0	1·34
10	1227	960	58	116	333	45	156	177	8·8	19·6	3·18	25·5	28·7	1·46
12	2043	1589	63	129	357	50	174	183	9·1	18·2	3·04	24·7	27·7	1·52
14·5	3323	2580	57	121	320	47	163	157	7·8	16·6	2·93	22·0	24·9	1·50
24	4763	3700	51	109	274	42	147	127	6·3	15·0	2·95	19·7	22·6	1·51
33	4963	3850	57	120	276	47	161	115	5·7	12·1	2·57	19·4	22·0	1·82

(i) = Optical density $\left(\log_{10}\frac{I_0}{I}\times 1000\right)$ at 700 mμ., of the *culture*.

(ii) = (i) $\times \frac{700}{0{\cdot}9} \times \frac{1}{1000}$.

(iii, iv, v) = Optical densities of *washed suspensions* in water.

(vi) = (iv) $\times \frac{350}{0{\cdot}9} \times \frac{1}{1000}$. (vii) = (iv) $\times \frac{350}{260}$. (viii) = (v) − (vii).

(ix) = (viii) $\times \frac{1}{20}$. (x) = $\frac{(\text{ix})}{(\text{vi})} \times 100$.

$\text{Log}_2\, m$ is obtained from (ii) and k_{350}/k_{700} from (iv)/(v).

the lag phase apparently differed by over an hour in the two experiments, the course of the changes in % NA was very nearly coincident. The figures for one of these experiments are set out in Table 2 to illustrate the method of calculation. The values of the ribonucleic and desoxyribonucleic acid contents of the same samples of cells are shown for comparison. These were kindly obtained by Jennifer Moyle, using an adaptation of the Schmidt & Thannhauser phosphorus method (Stephenson & Moyle, 1949). The latter values for the ribonucleic acid and total nucleic acid contents of the cells are also plotted in Fig. 6 (crosses corresponding to open circles). In these experiments, the inoculum was prepared by growing one Roux bottle sown with 5 ml. of *M. pyogenes* (suspension density in medium 2 mg./ml.) for 48 hr. at 25°, when the density reached about 0·85 mg./ml. Two litres of medium, stirred and aerated in a 5 l. rotated flask, was sown with 100 ml. of the inoculum and samples subsequently taken, beginning with a volume of 200 ml. and diminishing

as the dry weight of the culture increased, so as to keep the dry weight of each sample at about 30 mg. From each sample, 5 ml. were taken immediately for estimation of $\log_{10}(I_0/I)$ at 700 mμ., where the small absorption of the medium was readily compensated by a blank estimation, and the dry weight calculated on the basis of $k = 0{\cdot}90$. The remainder was centrifugally washed three times in distilled water and made up to a volume of just over 11 ml. Of this, 1 ml. was

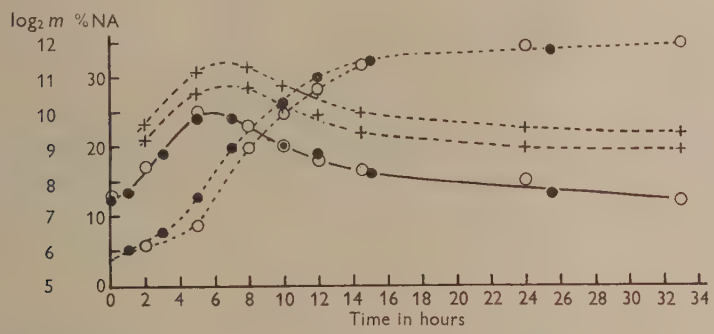


Fig. 6. The percentage by weight of 'nucleic acid' estimated throughout a normal growth of *M. pyogenes*. Open (- - ○ - - ○ - -) and closed (- - ● - - ● - -) circles on broken (- - - - - - -) lines, $\log_2$ (dry weight in μg./ml.) indicating phase of growth, duplicate experiments; open (— ○ — ○ —) and closed (- - ● - - ● - -) circles on full (————) line, corresponding percentages by weight of 'nucleic acid' in the cells estimated by present method. Erect (- - + - - + - -) crosses on broken lines, estimation of ribonucleic and total nucleic acid by the method of Schmidt & Thannhauser (1945) corresponding to open circles. Open circles (○ ○ ○) and crosses (+ + +) correspond to figures of Table 2.

suitably diluted (usually 1/100), and used for the spectrophotometric estimations, while 10 ml. were pipetted into 2 ml. of 30% trichloroacetic acid and reserved for chemical estimation.

The absolute values of the percentage by weight of 'nucleic acid' obtained by the present method are about 30 % lower than those given by the Schmidt & Thannhauser phosphorus technique. The ratio of the two values, however, shows a standard variation of only 10 %.

A closer examination now in progress, of the results given by the Schmidt & Thannhauser method, has shown that the phosphate values of the 'ribonucleic acid fraction' are too high to be accounted for on the basis of a tetranucleotide structure. A typical sample of *M. pyogenes* (density at harvesting from Roux bottle, 1 mg./ml.) gave the values of Table 3, based upon phosphorus and ultra-violet absorption estimations on the fractions. The absorption measurements on the extracts agreed well with the value of 18·5 % for the % NA found on the cell suspension. It will be noticed that this value includes the acid-soluble fraction. Although the acid-soluble purine-pyrimidine fraction is omitted in the estimation of total nucleic acid by the Schmidt & Thannhauser method, the figure for the total 'nucleic acid' obtained either on the intact suspension or on the fractions by the ultra-violet absorption measurements was 30 % lower than that obtained by the phosphorus estimations. The absolute

Table 3. *Comparison of phosphorus and ultra-violet estimation of 'nucleic acid' in Schmidt & Thannhauser fractions of* Micrococcus aureus

Fractions by Schmidt & Thannhauser (1945)	Percentage by weight of 'nucleic acid'		
	By absorption at 260 mμ. (coeff. 200)	By phosphorus content	Direct on cell suspension
Acid soluble	2·7	—	—
Ribonucleic acid	11·4	21·3	—
Desoxyribonucleic acid	4·0	4·4	—
Total	18·1	25·7	18·5

discrepancies between the % NA values and the values for the percentage of total nucleic acid given by the Schmidt & Thannhauser technique thus appear to be due rather to inaccuracies in the phosphorus method than in the present ultra-violet absorption method. The absorption coefficient of 200 assumed for the 'nucleic acid' is probably a lower limit. When a value near the probable upper limit of 250 is used, the % NA values are lowered by 20 %.

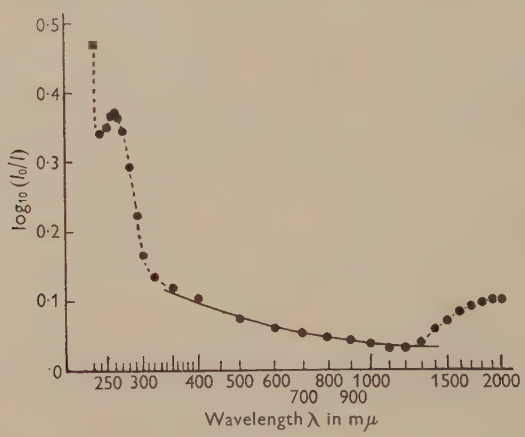


Fig. 7. Absorption and scattering spectrum of *Esch. coli* (strain H) in phosphate buffered saline, suspension density 46 μg./ml. Full line, the inverse law.

The transmission spectrum of a washed suspension of *Escherichia coli* (strain H) in phosphate buffered saline is shown in Fig. 7. As in the case of the micrococcus, the scattering spectrum between 1000 and 350 mμ. approximated closely to the simple inverse law. The estimation of % NA of the cells, harvested in the late log phase of growth, gave 22·8 % when the same method of computation was used as for the micrococcus.

DISCUSSION

The photometric estimation of substances inside cells, whether by microscopic observations upon individual cells or by spectrophotometric examination of cell suspensions, depends upon certain assumptions of questionable validity.

It is assumed in the method described in this paper that the Beer-Lambert law is applicable to the 'nucleic acid' of the cell suspension as if it were in homogeneous solution in the same volume. The 'nucleic acid', of course, includes all the substances showing the purine-pyrimidine absorption at 260 mμ., and we have referred to it as 'nucleic acid' or NA in order to avoid confusion. The part of the NA which is uniformly distributed in the cells may reasonably be expected to obey the Beer-Lambert law, but the applicability of the law to the variable proportion which is present in the cells as granules is very uncertain. Moreover, the choice of the absorption coefficient of the NA, as present in the cells, is difficult, because the NA is a complex mixture, the concentration of some components of which is high, and there may be some degree of orientation. Malmgren & Heden's value of 200 for the percentage decadic absorption coefficient has been accepted mainly on the ground that it is a good round number. Under the circumstances, it would not be consistent to attempt to correct for the small absorption of other substances such as aromatic amino-acids at 260 mμ.

It is further assumed that the absorption and scattering spectra contribute additively to the transmission spectrum of the suspension; and that the scattering contribution in the near ultra-violet, where the cells absorb strongly, can be obtained by extrapolation of the inverse scattering law from the visible region where there is little light absorption. These two assumptions are supported by the observations that a change of refractive index of the suspension medium which causes a fivefold change in the scattering coefficient of *Micrococcus pyogenes* has little or no effect either upon the form of the scattering spectrum or upon the estimated NA absorption; and that there is a linear relationship between the suspension density and the estimated NA concentration over a broad range. No other direct evidence has been obtained in support of the basic assumptions, and no attempt will be made to argue against their doubtful validity.

The occurrence of a minimum in the scattering spectrum of the suspension of *M. pyogenes* is in agreement with the theoretical considerations of LaMer (1943) on smokes, and with the experimental observations of Bailey (1946) on suspensions in liquids. For *M. pyogenes* harvested during the major part of the log phase of growth, the value of the optical density of the washed suspension between 1000 and 350 mμ. is inversely proportional to the wave-length. Significant deviations occur, however, when the cells are harvested at phases of growth when they are larger or smaller than the logarithmically growing cells. At the phases of growth where the cells are larger the spectra are flatter, while, when they are smaller the spectra are steeper, in conformity with observations on oleic acid and glass suspensions of different particle sizes.

During a normal growth cycle, the value of k_{350}/k_{700} has been found to be equal to $1 \pm 11\%$, so that the error introduced into the determination of the scattering contribution to the optical density at 260 mμ. from the value at 350 mμ., on the assumption that $k_{260} = k_{350}$, may be considered to be $\pm 5\%$; and since the scattering and absorption contributions to $\log_{10}(I_0/I)$ at 260 mμ. are approximately equal, the error introduced into the estimation of % NA is

also $\pm 5\%$. No correction has been applied for this error, but it should be borne in mind that a correction may become necessary if the physiological conditions of the cells induce abnormal size variations, recognized by abnormal deviations of k_{350}/k_{700} from unity. Indeed, the measurement of the slope of the scattering spectrum might form the basis of a quantitative method of measuring changes of mean cell diameter.

In addition to changes in k_{350}/k_{700} during growth, there are variations in the absolute value of k_{350} which are probably caused in part by factors other than changes in cell size. The value of k_{350} shows a drop of about 10 % at the approach to the stationary phase of growth and later a rise to the initial value. This variation introduces a 10 % error in the value of % NA if k_{350} is assumed to remain constant. The error may be avoided by determining k_{350} gravimetrically on the washed suspensions used for the photometric measurements, a procedure which becomes essential if the scattering characteristics of the cells are suspected of being abnormal. Attention should, perhaps, be drawn to the fact that turbidimetric methods of estimating dry weight are generally liable to errors of 10 % or more unless calibrations are made with cells in corresponding physiological states (Longsworth, 1936; Hershey, 1939).

In view of the nature of several of the assumptions involved in the evaluation of % NA, it was not surprising to find that the absolute values fell about 30 % short of those obtained by the Schmidt & Thannhauser phosphorus method. The subsequent finding that most of this discrepancy appears to be due to the erroneously high values of the phosphorus method was quite unexpected, and is at present under investigation. The ratio of the two values, however, shows a standard variation of only 10 % under the normal range of physiological conditions obtaining during the growth of the culture.

The single experiment on *Escherichia coli* (strain H), harvested towards the end of the log phase, indicates that the same method of measuring % NA may be applied as has been used for *M. pyogenes*. The % NA in *Esch. coli* measured in this way is nearly the same as in the micrococcus at the same phase of growth. It is suggested that the present method of estimating total 'nucleic acid' may be of general applicability when the necessary exploratory data have been obtained.

I am indebted to Dr Gale and the members of the Medical Research Council's Unit for Chemical Microbiology at Cambridge for valuable help and criticism, and to the Medical Research Council for a personal grant.

REFERENCES

Bailey, E. D. (1946). Particle size by spectral transmission. *Industr. Engng Chem.* (Analyt. ed.), **18**, 365.

Hershey, A. D. (1939). Factors limiting bacterial growth. 4. The age of the parent culture and the rate of growth of transplants of *Escherichia coli*. *J. Bact.* **37**, 285.

LaMer, V. K. (1943). Progress report on 'Verification of mie theory—calculations and measurements of light scattering by dielectric spherical particles'. *Report OSRD*, no. 1857.

Longsworth, L. G. (1936). The estimation of bacterial populations with the aid of a photoelectric densitometer. *J. Bact.* **32**, 307.

MALMGREN, B. & HEDEN, C. (1947). Studies of the nucleotide metabolism of bacteria. 1. Ultraviolet microspectrography as an aid in the study of the nucleotide content of bacteria. *Acta Path. microbiol. Scand.* **24**, 417.

MITCHELL, P. (1949*a*). Some observations on the mode of action of penicillin. *Nature, Lond.*, **164**, 259.

MITCHELL, P. (1949*b*). A new technique for stirred aerated culture. *Nature, Lond.*, **164**, 846.

SCHMIDT, G. & THANNHAUSER, S. J. (1945). A method for the determination of desoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. *J. biol. Chem.* **161**, 83.

SCHNEIDER, W. C. (1945). Phosphorus compounds in animal tissues. 1. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. *J. biol. Chem.* **161**, 293.

STEPHENSON, M. & MOYLE, J. M. (1949). Nucleic acid metabolism of *Escherichia coli*. *Biochem. J.* **45**, vii.

(*Received* 17 *December* 1949)

A Versatile Fermentation Sampling Arrangement

By N. G. HEATLEY

The Sir William Dunn School of Pathology, University of Oxford

SUMMARY: The sampling tube from the fermentation vessel is connected to a tube passing loosely through the specially fitted cap of a standard screw-capped bottle. By means of a mouthpiece suction can be applied so that a sample of fluid is drawn over into the bottle, which is then replaced by a similar empty sterile bottle. By fitting an extension of the tube to pass to the bottom of the bottle, and by blowing instead of sucking, fluid in the bottle may be driven over into the fermentation vessel. The device may be used for certain other operations such as removing or transferring sterile solutions or suspensions.

Among the desirable features of any arrangement for withdrawing samples from a pure culture fermentation are the following: (1) The risk of contamination of the main fermentation—and in some cases of the sample also—should be as small as possible. (2) There should be no dead space. If there is, the first part of the sample must be taken separately and rejected, or the sample must be large in relation to the volume of the dead space. (3) It should not be necessary to interrupt aeration or stirring of the fermentation. (4) The arrangement should not be unduly complicated or expensive.

It is believed that the device described below fulfils these requirements better than many of the sampling arrangements in common use. It has been useful for certain other aseptic operations.

CONSTRUCTION

This will be understood from the sectional drawing (1) in Fig. 1.

A screw-cap *a*, fitting a standard 1 or 4 oz. bottle *h*, is attached to a glass or metal tube *b* of the same outside diameter by a rubber sleeve *c*. The top of the tube *b* is closed by a rubber bung *d* pierced with two holes. One of these carries a straight tube *e* which passes loosely through a hole in the centre of cap *a* and its washer. The lower end of tube *e* is tapered slightly. The second hole in bung *d* carries a short bent tube *f* connected by rubber tubing to the mouthpiece *g*.

Tube *b* is packed with cotton-wool by raising the bung slightly and feeding in the cotton-wool as a loose rope. As it is fed in, in a helical fashion, it is tamped down with a rod. Finally bung *d* is pressed home and *e* is adjusted so that its tip is 22 mm. below the rubber washer in cap *a*. Once filled, the apparatus may be used again and again without repacking. It is, of course, sterilized with a bottle *h* in position, as shown.

METHODS OF USE

As a fermentation sampler. The top end of tube *e* is connected to a tube passing into the fermentation vessel and ending below the level of the fluid in it. (During the initial sterilization of the whole set-up it may be desirable to clamp

off this connexion.) To take a sample, slight pressure is applied to the mouthpiece *g* in order to drive out any medium from the dead space of the tube. Suction is then applied till the desired amount of medium has come over into bottle *h*. If the latter is below the level of the liquid in the fermenter or if the pressure in the latter is greater than atmospheric, it may be necessary to break

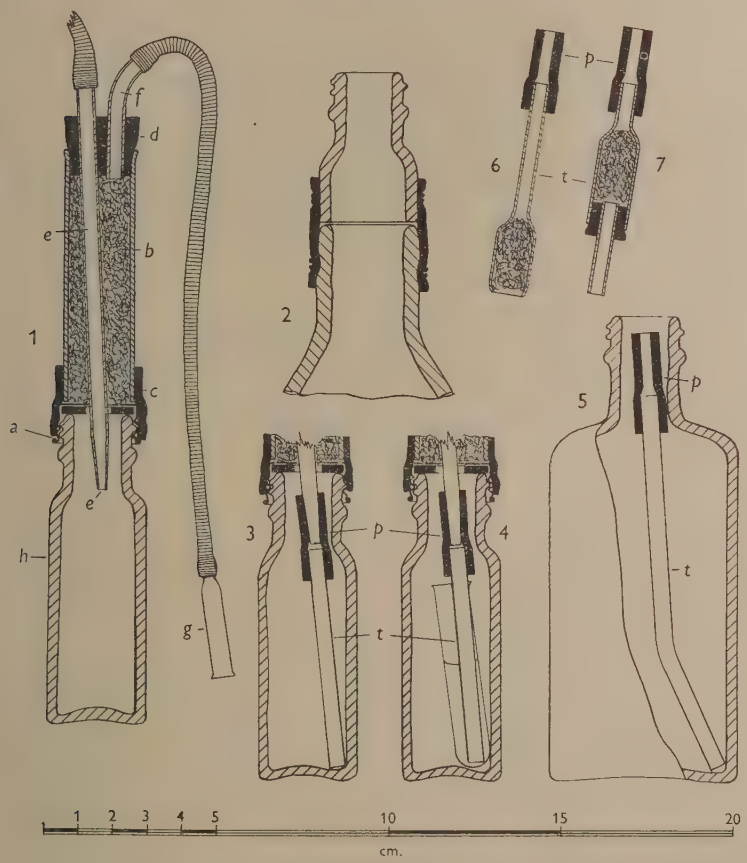


Fig. 1

the syphon by applying momentary pressure at *g*. A clean sterile bottle is now substituted for *h*, which in turn is closed with the cap from the clean bottle. This changing, which takes 4 or 5 sec., is the only time during which there is exposure to contamination.

The size of the sample may be varied from a few drops to a bottleful, and it is as easy to attach a 4 oz. as a 1 oz. bottle. Still larger samples may be taken with the device shown at (2) in the figure, consisting of the upper half of a 1 oz. screw-capped bottle attached to the neck of a larger bottle or flask by a rubber sleeve.

For adding sterile solutions or inocula to a fermentation vessel. The solution to be added is made up and sterilized in the amount to be added in 1 or 4 oz. screw-capped bottles. The bottles contain, or have added to them, pieces of glass tubing *t* the upper ends of which bear short pieces of pressure tubing *p*. If separately sterilized, for example in a plugged test-tube, the rubber should be at the *bottom* of the tube so that when tipped into the bottle of fluid to be added the rubber part is at the *top*. To transfer the fluid, the bottle containing it (and a tube *tp*) is substituted for *h*, the tapered tip of tube *e* being guided into the pressure tubing *p*. Mouth pressure is then applied at *g* until all the fluid has been driven over. For a 4 oz. bottle it is an advantage if the tube *t* is slightly bent ((5) in Fig. 1). For adding very small amounts of fluid the arrangement shown at (4) may be used; but with any type of container quantitative transfer may be achieved by blowing the fluid over then sucking back some of the fermentation liquid to rinse the container. This procedure has been useful for making good the losses due to evaporation in simple small aerated cultures by the daily addition of distilled water.

For inoculation of the main fermentation. Cultures can be grown in fluid medium in any suitable screw-capped bottle then blown over as described above. The organism can also be grown on a slope or a thin layer of solid medium and suspended by shaking with water, with or without glass beads. The suspension is then blown over in the usual way. If desired, *t* may be modified, as at (6) or (7) in Fig. 1, so that the fluid transferred is filtered through glass- or cotton-wool.

Direct inoculation from a main fermentation vessel is also easily carried out by withdrawing one or more samples and transferring each or a part of each to each of the vessels to be inoculated.

For the sterile removal of supernatants, etc. Outlet tube *e* is connected by a short length of rubber tubing to a glass tube of which the lower end is drawn out to a tip and bent nearly to a right angle. The tube is kept sterile by enclosure in a boiling- or test-tube, through the plug of which it passes. The protecting tube is chosen of such a size that the plug will fit snugly into the centrifuge bottle containing the material to be separated, so that as the latter is withdrawn the air taking its place will be filtered through the plug. After the supernatant has been withdrawn the sediment may be washed with any desired liquid by attaching a bottle of it to screw-cap *a* and blowing it over. The bent tip of the delivery tube enables a powerful swirling motion to be set up. If the sediment is not readily dispersed in the washing fluid this can be facilitated by sucking the suspension backwards and forwards several times. The supernatant or the resuspended sediment may be dispensed direct into a number of sterile bottles with the minimum chance of contamination.

(*Received* 19 *December* 1949)

The Differentiation of Certain Genera of Bacteriaceae by the Morphology of the Microcyst Stage

By K. A. BISSET

Department of Bacteriology, University of Birmingham

SUMMARY: The microcysts or resting cells of Bacteriaceae have three typical forms, which are regular and recognizable, and by which the Family may be divided into three groups.

The first type of microcyst is small, oval and eccentrically nucleate; it is typical of *Bacterium coli*, and *Proteus*, *Pseudomonas* and *Salmonella* spp. with the exception of *Salm. typhi* which forms a very large, oval microcyst, with a central nucleus. The microcysts of *Shigella* spp. resemble those of *Salmonella typhi*. *Bacterium aerogenes* produces large, spherical or oblong microcysts with a small, central nucleus.

It has for long been bacteriological dogma that the members of the large group of Gram-negative, non-sporing bacteria are morphologically almost indistinguishable, apart from the presence or absence of flagella or capsules, and in some cases, the arrangement of flagella. While this is approximately true of the vegetative cell, the morphology of the resting cell, which in this family closely resembles the microcyst of myxobacteria (Bisset, 1949, 1950), appears to provide a criterion of considerable systematic value. It has already been observed that a striking difference exists between the microcyst of *Bacterium coli* and *Bact. aerogenes*, the latter being much larger. The present paper is intended to extend these observations and to indicate the degree to which they may be correlated with other criteria used in classification.

METHODS AND MATERIALS

In almost every case the bacteria examined were newly isolated. I consider that such delicate, morphological comparisons should not be made upon material which has been cultivated artificially for longer than is absolutely necessary. Where this ideal was difficult to achieve, as in the case of *Salmonella typhi*, observations made originally upon newly isolated strains were confirmed, as far as possible, upon stock cultures of the same species.

Cultures were made upon nutrient agar. They were incubated at 37° for 24 hr. and afterwards stored at 20°. Mature resting cells appeared in such cultures after a period varying from a few days to 6 weeks or more. Preparations were stained by the acid-Giemsa or methylene-blue eosin techniques (Bisset, 1950), and were mounted in water for examination.

RESULTS

Differentiation of Bacterium coli *from* Bacterium aerogenes

Fifty strains of lactose-fermenting bacteria were isolated from human and animal faeces, soil, and plant surfaces. Of these strains twenty-seven were indole-positive and Voges-Proskauer negative, motile and non-capsulated

Twenty-three were indole-negative and Voges-Proskauer positive, non-motile and capsulated. The former will hereafter be referred to as *Bact. coli*, the latter as *Bact. aerogenes*. One strain of *Bact. coli* differed from the remainder in the production of a voluminous slime layer. This, however, was not a capsule according to the criteria of Klieneberger-Nobel (1948), as it was irregular in outline. This organism, like the other *coli* strains, was motile, which I consider, in this Order, to be synonymous with flagellated.

The morphology of the microcyst stage was in complete accordance with this grouping. The microcysts of *Bact. coli* were relatively small, elongate ovals, and the nucleus stained eccentrically (Fig. 1 A; Pl. 1, figs. 1, 2). They were approximately half the size of young, vegetative bacteria of the same species. The microcysts of *Bact. aerogenes* were from 1·5 to 3 times as great as those of *Bact. coli*, spherical or oblong in shape, often retaining the capsule to some extent, and with small, central nuclei (Fig. 1 B; Pl. 1, figs. 3, 4). This difference is so striking that these two species may readily be distinguished by the simple, microscopic examination of mature cultures. In order to check this important point, I examined these fifty strains, stained and numbered by another person, and identified them correctly by this means alone. It was even possible to detect preparations made from mixed cultures, and containing both *coli* and *aerogenes* type microcysts. In three cases, hitherto unsuspected, contamination of cultures was revealed in this way, and their unusual biochemical behaviour explained. A possible source of error is excessive age of cultures. All types of microcyst may be distorted by desiccation and become difficult to identify.

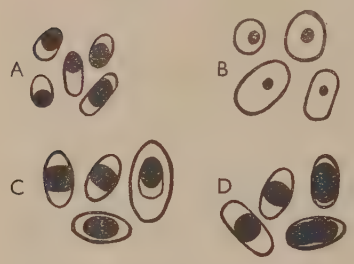


Fig. 1. Microcysts of Bacteriaceae. A, *coli* type (*Bact. coli*, *Salmonella*, *Proteus*, *Pseudomonas*). B, *aerogenes* type. C, *S. typhi*. D, *Shigella*.

About a dozen strains of lactose-fermenting bacteria, derived from the faeces of animals and from vegetation, failed to conform exactly to either of these groups. Some produced indole, although non-motile; in others the macroscopic appearance of the colony was markedly unlike that of typical *coli* or *aerogenes*, and the vegetative cell was small and irregular in form. Most of these possessed large microcysts with central nuclei, like *aerogenes*, but spherical, oval or irregular in shape.

Salmonella

Eighteen strains of *Salmonella* were examined, of which twelve were newly isolated. These included *Salm. paratyphi* B and C, and *Salm. enteritidis*. All possessed the type of microcyst characteristic of *Bacterium coli*, i.e. small, oval, and eccentrically nucleate. The morphology of the eight strains of *Salmonella typhi-murium*, which were examined, was unusual. The typical primary nucleus (Bisset, 1950) was not seen at any stage; the vegetative nucleus was

large and irregular. This condition is more often found in *Bacterium aerogenes* than in *Bact. coli*, but the microcysts of *Salmonella typhi-murium*, where they occurred, appeared to be of the *coli* type.

Salmonella typhi *and* Shigella

The microcyst of *Salm. typhi* was different from that of other salmonellas, or indeed from either of the previously mentioned types. Fourteen strains were examined; six had been in laboratory culture for only a few months, eight were stock cultures. In the newly isolated strains, and in the majority of the stock cultures (probably the most recently isolated), the microcysts were approximately the same size as those of *Bacterium aerogenes*, but were oval in shape and had large, central nuclei; some microcysts were relatively enormous (Fig. 1 C; Pl. 1, figs. 5–7). These exceptionally large cells possessed nuclei which were, correspondingly, so large that their vesicular structure was clearly visible (Pl. 1, figs. 6, 7). Three of the stock cultures failed to produce recognizable microcysts.

The appearance of the microcysts of *Shigella* which were examined (ten *Sh. flexneri*, six *Sh. sonnei*, one *Sh. schmitzii* and one *Sh. alkalescens*), all of which were newly isolated, was very similar to that of the smaller microcysts of *Salmonella typhi*, but the nucleus was often irregular in shape (Fig. 1 D; Pl. 1, fig. 8). In cultures of *Shigella sonnei* occasional microcysts were irregular in outline, but were recognizable as representatives of the same morphological type.

Proteus *and* Pseudomonas

Five newly isolated strains of *Proteus vulgaris*, six of *Pr. morganii*, four of *Pseudomonas fluorescens*, and five (four newly isolated) of *Ps. pyocyanea*, all possessed the *coli* type of microcyst.

DISCUSSION

In most branches of biology, the characteristics of the reproductive process are of primary importance in classification; it is justifiable to claim that the morphology of the microcysts of various genera of Bacteriaceae may provide evidence of their relationship. This criterion is already employed in the classification of myxobacteria.

On this basis, *Bacterium coli* and *Bact. aerogenes* are unlike, and, as their differences, both in the morphology of the vegetative bacterium and in their biochemical reactions, are more marked than their resemblances, the recognition of the genus *Aerobacter* is probably justifiable. *Bacterium coli* is morphologically identical with *Salmonella* (except *Salm. typhi*), and shares the habit of gut parasitism. The *coli* type microcyst is found also in *Proteus* and *Pseudomonas*, but in these genera the vegetative form is distinct. *Bacterium aerogenes* forms a group with a number of similar bacteria from the soil.

Salmonella typhi and *Shigella* resemble each other in their habitat in man, and in their failure to produce gas in the fermentation of carbohydrates. The latter characteristic is shared by the *Sh. pullorum* group, which are antigenically

related to *Salmonella typhi*, and like *Shigella*, are non-motile. Newly isolated cultures were unfortunately not available. The resemblance in microcyst form between *Salmonella typhi* and *Shigella* may be regarded as additional evidence of relationship.

REFERENCES

Bisset, K. A. (1949). The nuclear cycle in bacteria. *J. Hyg., Camb.*, **47**, 182.

Bisset, K. A. (1950). *The cytology and life-history of bacteria.* Edinburgh: Livingstone.

Klieneberger-Nobel, E. (1948). Capsules and mucoid envelopes of bacteria. *J. Hyg., Camb.*, **46**, 345.

EXPLANATION OF PLATE

(All stained by acid-Giemsa, except fig. 4, stained by methylene-blue eosin. Magnification ×3000.)

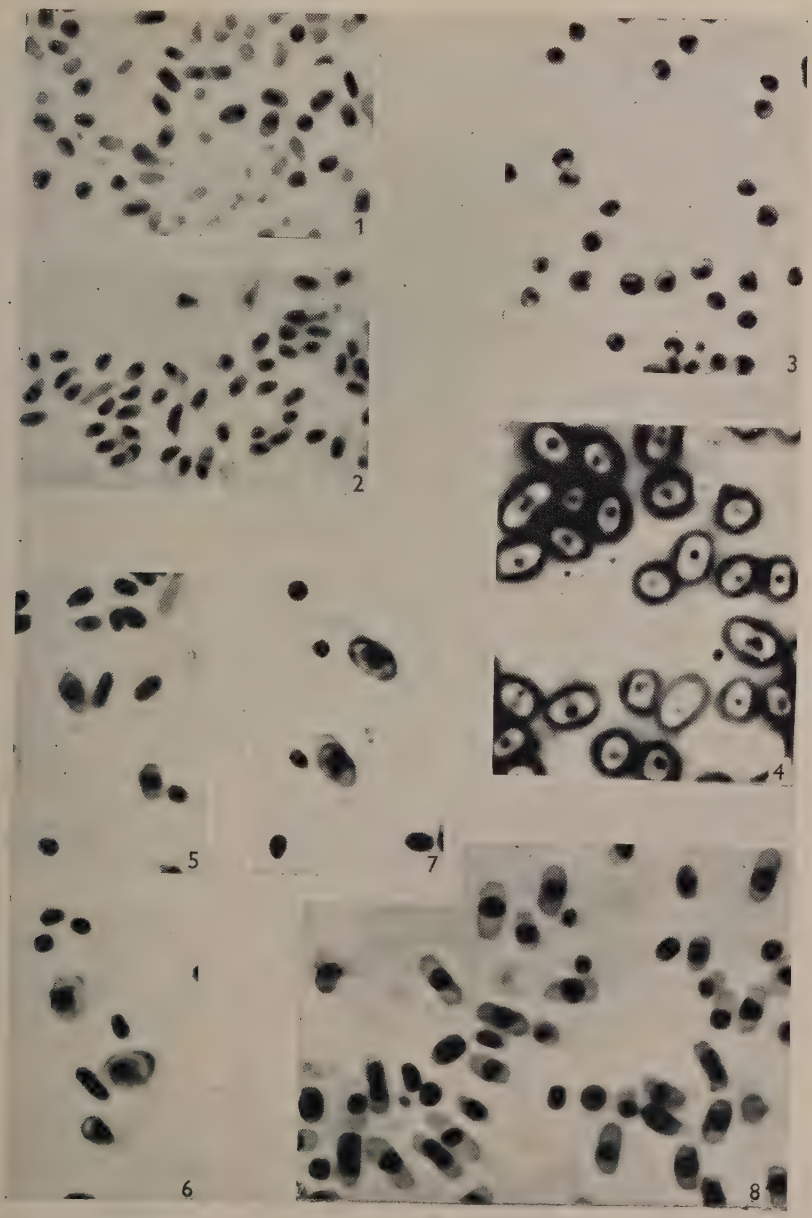
Figs. 1, 2. Microcysts of *Bacterium coli*. *Proteus*, *Pseudomonas* and *Salmonella* (except *Salm. typhi*) are similar.

Figs. 3, 4. Microcysts of *Bacterium aerogenes*.

Figs. 5–7. Microcysts of *Salmonella typhi*. These more closely resemble the microcysts of *Shigella* than of other species of *Salmonella*.

Fig. 8. Microcysts of *Shigella schmitzii*.

(*Received 23rd December*, 1949)



K. A. Bisset—The differentiation of certain genera of Bacteriaceae by the morphology of the microcyst stage. Plate 1

Marcescin, an Antibiotic Substance from *Serratia marcescens*

By A. T. FULLER and JEAN M. HORTON

National Institute for Medical Research, Mill Hill, London, N.W. 7

SUMMARY: Marcescin, a thermostable polypeptide isolated from a strain of *Serratia marcescens* inhibits the growth of *Corynebacterium diphtheriae* at 0·01 μg./ml. and *Staphylococcus aureus* at 0·1 μg./ml. It is produced in good yield in 18–36 hr. at 24° in an aerated ammonium citrate glycerol medium. It was very toxic to mice and in sublethal doses failed to protect mice against sensitive organisms. It is very strongly adsorbed by bacteria so it is not possible to say whether it is bacteriostatic or bactericidal in high concentration.

Serratia marcescens, formerly known as *B. prodigiosus*, is a red-pigmented non-sporing Gram-negative bacterium. There are several records of antibiotic activity in the *Serratia* group, but practically all observers (e.g. Hettche, 1932) have ascribed it to prodigiosin, the red pigment. Eisler & Jacobsohn (1935) and Lichstein & van der Sand (1946), however, found evidence of a colourless, heat-stable antibiotic, which they did not isolate.

The substance described below, marcescin, is a colourless thermostable polypeptide having a powerful inhibitory action on the growth of *Pasteurella septica*, and on *Corynebacterium diphtheriae* and other Gram-positive bacteria. It was obtained from a bacillus (strain 82 B) isolated from soil and identified as *Serratia marcescens*.

EXPERIMENTAL

Isolation of the organism. After many trials the following technique was adopted for isolating antagonistic organisms from soil. Suitably diluted soil samples were plated and colonies allowed to develop for 4 days at 23°. The indicator organism (*Staphylococcus aureus* or *Escherichia coli*) was added in 1·5 % nutrient agar pipetted on to the surface of the plates, which were incubated overnight at 37°. Strains antagonistic to the indicator organism were isolated and tested against eight to ten species of pathogenic bacteria.

Determination of antibiotic titre. The serial dilution method was employed using 200 μl. of broth seeded with 10 μl. of a 1/500 dilution of an overnight broth culture of *Staphylococcus aureus*. The results were often inconsistent owing to the appearance of resistant organisms with a granular type of growth. Tests were therefore made in duplicate. A unit of marcescin activity was provisionally defined as the amount which in 1 ml. was able to prevent the overnight growth of *Staph. aureus*. The number of units/g. is therefore the limiting dilution at which the material was active.

Description of the antibiotic producing Serratia *strain*

The bacillus (82 B) is motile, Gram-negative, and produces a pink pigment in broth cultures in 2–3 days at 23°. Twenty-four hour colonies on agar are circular with an entire edge, a glassy surface, and a yellowish coloration later becoming pink. It grows moderately well without forming pigment at 37°. The optimum temperature for growth and pigment production is about 23°. On nutrient agar the organism throws white variants which breed true for several subcultures and produce antibiotic as efficiently as the pigmented strains. Gelatin cultures liquefy in 1–2 days and a milk medium is coagulated in 2 days; acetylmethylcarbinol positive, indole negative. In peptone water acid and gas are produced from glucose, sucrose, raffinose, fructose, maltose, galactose, xylose and mannitol, but not from lactose, arabinose or dextrin. Lactose is fermented in absence of peptone, e.g. in aqueous solution or milk. These properties place the organism in the family Enterobacteriaceae, tribe Serrateae, genus *Serratia* (Bergey, 1948).

The organism differs culturally from *S. piscatorum*; from *S. plymuthicum* in failing to produce gas from lactose; and from *S. kilensis* in its ability to form acetylmethylcarbinol and in absence of pigmentation at 37°. The ultra-violet absorption spectrum of the pigment from strain 82 B in amyl alcohol showed a closer similarity to that from *S. marcescens* N.C.T.C. 4612 than to that from *S. kilensis* N.C.T.C. 4619.

The pink pigment of 82 B is sparingly soluble in water and soluble in ethanol, ether and chloroform. In this property, and in its failure to produce a conspicuous pellicle on gelatin (but see below), its failure to produce indole, its liquefaction of gelatin and its coagulation of milk (but see below) the organism resembles *S. marcescens* (Bergey, 1948). It also has affinities with *S. indica*, but it is doubtful whether *S. indica* is really distinct from *S. marcescens*. *S. indica* is stated by Breed & Breed (1926) to differ from *S. marcescens* in producing an orange pellicle on gelatin, in growing in a KCl urea glucose medium, and in producing a gas which is solely CO_2 from glucose (*S. marcescens* produces some hydrogen as well).

S. indica (N.C.T.C. 2847), *S. marcescens* (N.C.T.C. 1377, 2302, 2842, 2847 and 3804), and 82 B, were examined by these and other tests to see whether 82 B resembled *marcescens* or *indica* more closely. In a 15% gelatin stab made up with meat extract all cultures grew well with liquefaction in 1–2 days at 23°. Pellicles occurred at some stage up to 7 days in all cultures except 82 B and there was no distinction between *marcescens* and *indica*. N.C.T.C. 1377 had an orange pellicle at 2 days which later became pink, but the *S. indica* culture was never pigmented. None of the cultures could be made to grow from an inoculum of washed cells in a medium containing 0·5% KCl, 1% urea and 1% glucose.

Pederson & Breed (1928) used large-scale cultures and gas analysis in showing that *S. marcescens* produced hydrogen as well as CO_2, whereas *S. indica* produced only CO_2 on an inorganic salt glucose medium. We failed to get gas from any of the cultures on an inorganic medium in Durham tubes. In glucose

peptone water, 82 B rapidly produced gas, half of which was absorbed by NaOH. Of the other cultures only 1377 and 2302 gave minute amounts of gas which were not completely absorbed by NaOH. In this 82 B resembles *S. marcescens.*

None of these tests enabled us to place 82 B definitely in the *marcescens* or *indica* species. The species *marcescens* is, however, heterogeneous. Reed (1937) by agglutination tests put N.C.T.C. 1377, 2302 and 2446 into one group, and N.C.T.C 2842 and 3804 into another. Taking the strains investigated by Reed, we found that fermentation of raffinose and lactose, reduction of ammonium molybdate to a blue compound (Marchal & Girard, 1947) and intensity of pigment formation divides them into the same two groups (Table 1). The strains in both groups are strongly proteolytic. *S. indica,* N.C.T.C. 2847, differs from Reed's first groups in failing to clot milk under the test conditions and in poverty of pigment. It is, however, closer to this section group than to Reed's group 2, which differs from group 1 in raffinose fermentation, molybdate reduction and pigment formation. *S. marcescens,* N.C.T.C. 4612, is an intermediate strain, fermenting raffinose slowly and being weakly proteolytic.

Table 1. *Biochemical reactions of strains of* Serratia marcescens *and* Serratia indica

Culture no. N.C.T.C.	Agglutination by antiserum to 2446*	Raffinose fermentation	Lactose fermentation	Molybdate reduction	Gelatin liquefaction	Milk† clotting	Pigment‡ formation
1377	+	−	−	−	++	++	++
2302	+	−	−	−	+	++	++
2446	+	−	.	.	+	.	++
2842	−	+	+	+	+	+	−
3804	−	+	+	+	+	++	±
4612	.	±	−	.	±	−	−
2847§	.	−	−	−	+	−	−
82 B	.	+	±	−	+	−	±

* Reed (1937). † In 3 hr. at 45° by 3–7 days' culture fluid.
‡ 7 days' broth culture. § *S. indica.*

Strain 82 B corresponds neither to Reed's strains, nor to *S. indica.* Neglecting 4612, the property which differentiates all the strains of *S. marcescens* from the strain of *S. indica* used is that of the rapid clotting of milk by culture filtrates. Culture filtrates from 82 B fail to do so, and the organism thus resembles *S. indica* in this respect. All the members of the family when grown in a milk medium clot it.

There seems little justification for division of the two species, since *S. marcescens* is heterogeneous (Reed, 1937), and Bergey (1948) suggested that *S. indica* and *S. marcescens* are variants of the same species. We have classified 82 B as *S. marcescens.*

Production of antibiotic

The active substance is stable to boiling and is present in the supernatant fluid of boiled cultures. *S. marcescens* is an aerobic organism and needs a good supply of air for rapid growth and adequate antibiotic production. In

preliminary experiments, shallow layers (0·5 cm.) in medicine bottles were used.

Effect of medium

Hartley broth. Maximum yields of 16–160 u./ml. were obtained in 3–5 days at 23°. The pH of cultures fell to 6·8 on the first day and rose on the second and third days to 8·0 or 8·5, but the change gave no indication of the point of maximum yield. The turbidity of the culture, however, tended to decrease at maximum titre. Antibiotic was not produced at temperatures above 25°, although growth was good. The antibiotic disappeared from sterilized broth cultures in a few days even at 0°.

Chemically defined media. The first basal medium consisted of: $(NH_4)_2SO_4$, 20 g.; KH_2PO_4, 2 g.; $MgSO_4.7H_2O$, 0·5 g.; NaCl, 0·05 g.; to 1 l. with water; with an added carbon source. Maximum yields of about 64 u./ml. were obtained with 1% glucose, 2% mannitol or 0·5% glycerol, but 1% sodium citrate gave very low yields.

Supplements of 1% Amigen (a casein digest from Mead Johnson and Co., Evansville, U.S.A.) or beef juice, with 0·5% glucose yielded 64–256 u./ml. but little with mannitol or glycerol. A supplement of 1% Bactopeptone with mannitol or glucose suppressed antibiotic formation altogether. Addition of 1% of corn-steep liquor had no effect.

A medium in which the ammonium sulphate was replaced by ammonium citrate and containing 0·5 % of glycerol gave the most satisfactory results. In ammonium sulphate media, the pH fell to 4–4·5 in 2 days, while that with ammonium citrate kept at pH 6–6·2. The acidity was not the cause of the lower yield since repeated neutralization of the sulphate culture gave no better results, and when the medium was buffered at pH 7·6, no antibiotic was produced.

Trace elements had no effect or a deleterious one. $FeSO_4.7H_2O$, 10 mg./l., greatly diminished the yield of antibiotic, except in the presence of casein digests or citrates, when it was without effect. Neither $MnSO_4$, $ZnSO_4$, or $CaCl_2$ (10 mg./l.) affected the yield. When, however, the medium was prepared with tap-water instead of distilled water, the yield of antibiotic was very low.

Conditions of cultivation

Shallow layer cultures (1–3 mm.) reached maximum titre more quickly (1 day) than those in deeper layers (10–20 mm.; 3–4 days) and usually yielded more. Increase of the concentration of oxygen in the atmosphere to 50 % did not improve the yield. When inocula varying between 0·1 and 10% (v/v) of a vigorously growing culture were used, the largest inoculum gave much the best results, up to 1000 u./ml. at 42 hr. Rapid growth seemed necessary for high yields. Since shallow layers are unsuitable for larger scale preparation, volumes of 2 l. in 5 l. flasks, aerated through sintered glass disks were used. Six-hourly determinations of titre showed that with a 10% inoculum maximum yields of up to 2000 u./ml. occurred at 18–24 hr. at 22–24° (e.g. Table 2). With this short incubation period foaming was not troublesome.

Table 2. *Production of marcescin in a 3% ammonium citrate, 0·5% glycerol medium at 24°*

	Time (hr.)					
	6	12	18	24	30	36
pH:	6·2	6·0	6·2	6·2	6·1	6·2
Opacity*:	0·07	0·1	0·14	0·21	0·23	0·14
Pigment:	+	++	+	±	−	−
Units/ml.:	16	1000	1000	160	240	150

* Spekker absorptiometer reading for a 1/8 dilution.

In citrate glycerol media, neither pH nor opacity measurements helped to determine the point of maximum yield; nor did estimation of the residual glycerol in the medium, since maximum titres occurred when only a small part of the glycerol had disappeared. Attempts to assess the activity rapidly by testing the inhibition of reduction of methylene blue by *Staphylococcus aureus* were not satisfactory. Citrate cultures had the advantage over broth that after sterilization they lost little activity in 2 weeks at room temperature.

The preferred method for the production of the antibiotic was as follows. The medium consisted of: ammonium citrate, 30 g.; glycerol, 5 g.; KH_2PO_4, 2 g.; $MgSO_4.7H_2O$, 0·5 g.; distilled water 1 l.; and was sterilized by autoclaving at 10 lb. for 15 min.

The temperature of incubation was 22–24°. Cells from a 1 day slope were seeded into 40 ml. of medium which was incubated for 28 hr. in a shallow layer, and transferred to a flask containing 200 ml. of medium in a layer 10 mm. deep. Twenty-four hours later, the contents were added to 2 l. of medium in a 5 l. flask, which was aerated vigorously for 18 hr. The culture was sterilized by boiling, assayed, and kept until the next day, when it was processed if the titre was satisfactory.

During the course of the work, the titres of the ammonium citrate glycerol cultures fell from batch to batch, from the original 1000 u./ml. to 20 u./ml., and we were unable to regain the conditions necessary for high yields. We could find no reason for this decrease. Different batches of chemicals were tried. The age and temperature of incubation of the primary and secondary cultures were varied, and the stock culture, which had been passaged for 12 months, was replaced by a culture dried at the beginning of the work, but no difference was observed. Pigmented and achromogenic variants of the strain gave similar results.

There were indications that the strain had become more sensitive to the antibiotic. Originally it would grow in 1000 u./ml., but later it could withstand only 40–160 u./ml. A culture of *Serratia marcescens* was trained to grow in the presence of 1000 u./ml., with the intention of using it to prepare the antibiotic; but it lost its resistance at the first subculture in marcescin-free medium and so had no advantage over the stock culture.

Since the decrease in yield and in the resistance of the culture occurred together and were of the same order, an alternative explanation of the

phenomenon is that the conditions of testing had been inadvertently changed to make the antibiotic apparently less effective. No evidence for this could be found. The values recorded for the activity of the final product were obtained when low yields were being obtained, and hence under the possibly more stringent conditions of testing.

Antibiotic production by related organisms

S. kilensis N.C.T.C. 4619, and *S. indica* N.C.T.C. 2847, gave low titres in broth cultures. *S. marcescens* N.C.T.C. 4618, 2446 and 1377, yielded no antibiotic. Six strains of chromobacteria: *Chromobacterium amethystum* N.C.T.C. 4737, *Chromobact. violaceum* 2537, *Chromobact. orangium* 7185, *Chromobact. chocolatum*, *Chromobact. hiburnicum* 3073, and *Chromobact. viscosum* 2416, gave none or only traces. Four strains of *S. marcescens*, N.C.T.C. 3804, 2302, 4612 and 1377, and *S. indica* 2847, in a synthetic citrate medium, yielded only about 5% of the antibiotic titre of our strain. The conditions for antibiotic production by these strains were not examined exhaustively nor was the antibiotic produced identified with marcescin. Our strain of *S. marcescens* was more actively antibiotic than all other members of the species tested.

Extraction of the antibiotic

Broth cultures. The antibiotic could be extracted from broth cultures by adsorbing on blood charcoal, eluting with glacial acetic acid and precipitating with 4 volumes of ether. It was further purified by dissolving in water and precipitating by adjustment of the pH to 7·5.

Citrate cultures. The broth-extraction method failed with citrate cultures. The active substance was adsorbed by charcoal but it could not be removed from any of several grades of charcoal, even when the charcoal was pre-treated with a variety of reagents or after-treated with potassium cyanide solution.

Many other adsorbents were tried, including the oxides, hydroxides, carbonates, phosphates and silicates of Zn, Fe, Al, Ca and Mg; fullers' earth, Lloyd's reagent, kaolin, naphthols, benzoic acid, starch, coagulated protein and a variety of zeolites. Many of these adsorbed the active substance, but could not be made to yield it again in satisfactory amounts.

A wide variety of solvents and combinations of solvents, anhydrous or mixed with water, acids or bases was tried, but all failed to give satisfactory recovery. The solvents were formic, acetic and propionic acids, formamide and acetamide, dioxane, phenol, ethyleneglycol, propyleneglycol, glycerol, chlorhydrin, collidine, pyridine, piperidine, diethylamine, tripropylamine, acetone, methanol, ethanol, *iso*propanol and *n*-butanol.

Some success was achieved by adsorbing on acid-treated Decalso F, a cation exchanger (Permutit Ltd., London), and eluting with 0·1 N-HCl in water or 75 % ethanol, but the results were variable, and the method was abandoned. The citrate ion seemed to be the cause of the difficulty, and the following method was finally devised for removing it.

Boiled cultures of satisfactory titre were concentrated to one-tenth volume *in vacuo*, with a drop of tributyl citrate to prevent foaming. After acidifying to

below pH 1 (green to methyl violet) with 0·4 vol. of concentrated HCl, to convert all the citrate to free citric acid, 3 vol. of acetone were added to precipitate bacteria, inert protein and NH_4Cl. After allowing the precipitate to settle for 30 min., the clear supernatant was filtered, and the sludge filtered dry. An equal volume of acetone was added to the combined filtrates, the precipitate allowed to settle, the clear liquid siphoned off and the precipitate filtered dry. The filtrate contained the citric acid. The precipitate, which contained the antibiotic with much NH_4Cl, was dissolved in water equal in volume to the original concentrate and acidified to pH 3. The flocculent precipitate of low activity was removed and extracted with a little water. The combined extracts were adjusted to pH 9 with aq. ammonia (sp.gr. 0·880) to precipitate the antibiotic, and allowed to settle overnight. The precipitate was collected by siphoning and centrifuging, dissolved in water and adjusted to pH 3. A small inactive precipitate was removed and the liquid again adjusted to pH 9. The precipitate was collected by siphoning, centrifugation and finally filtration through a no. 3 sintered glass filter. The precipitate was thixotropic and the centrifuged deposit held large amounts of water, which could be removed only slowly. The precipitate was stirred with several volumes of acetone and filtered, and the process repeated with acetone and then ether and finally dried in the vacuum desiccator.

The yield of antibiotic was 65–100% of that demonstrable in the culture fluid. This figure is surprisingly high, and may be due in part to removal of antibiotic adsorbed on the bacteria.

A portion of the crude antibiotic preparation was soluble in 75 % aq. acetone; this was of low activity. By repeated extraction with neutral 75 % aq. acetone 1·11 g. of material of activity 700,000 u.g. was removed from 3·35 g., leaving a main fraction of 7 million u./g. This fraction gave a violet biuret reaction, a positive arginine test and a strong ninhydrin reaction after hydrolysis with acid. It contained 0·3% phosphorus, and 0·3% sulphur. The Molisch test was positive and corresponded to about 1·2% carbohydrate. It was necessary to eliminate the haemorrhagic polysaccharide obtained from *S. marcescens* by Hartwell, Shear & Adams (1943) as a possible cause of activity.

Further purification

Fractionation from organic solvents. Marcescin is soluble in aqueous organic solvents (e.g. 65% acetone) and portions are precipitated when the anhydrous solvent is added. No useful separation resulted from such treatment with a variety of solvents. Extraction with organic solvents in which the antibiotic was only partly soluble was also of little use, although the soluble fractions tended to be lower in activity. Table 3 gives typical results. Fractional precipitation by acetone from solutions in organic solvents was handicapped by the tendency of the substance to precipitate as a voluminous jelly. Marcescin (5 u./μg.) was fractionally precipitated by acetone from solution in various buffers. The precipitated portion was lower in activity the nearer the reaction was to neutrality, but no great purification resulted (Table 4).

Fractionation by isoelectric precipitation. Marcescin is soluble in dilute acid but the solution becomes more opalescent as it is neutralized. A 2 mg./ml.

Table 3. *Fractionation of marcescin from aqueous organic solvents*

		Fraction			
		Soluble		Insoluble	
Solvent	Mg. treated	Mg.	Activity (u./μg.)	Mg.	Activity (u./μg.)
66 % dioxane	14·2	8·9	2·7	5·3	4
50 % pyridine	7	1·7	3·2	4·9	3·2
90 % piperidine	16	3	0·5	•	•
72 % piperidine	*	3·4	3	•	•
45 % piperidine	*	4·8	6	7·4	3

* Insoluble portion from previous extraction.

Table 4. *Fractional precipitation of marcescin with acetone from buffer solutions*

Starting material contained 5 u./μg.

pH	Vol. acetone added	Precipitated portion as percentage of original		
		Activity u./μg.	Weight	Activity
5·5	4	1	25	5
3·5	5	2	10	4
3·0	4	3·6	3	2
1·5	5	4	30	24

solution adjusted to pH 6·8 and left overnight gradually developed a precipitate weighing *c.* 30% of the original. The activity, toxicity and absorption spectrum of this fraction were no different from those of the original material.

On the alkaline side of pH 9 only traces of material remained soluble, so fractionation was not possible in that pH range. When excess of NaOH is added to an acid solution, the marcescin remains in solution for some time but precipitates after a few hours.

The isoelectric precipitate is soluble in 65% acetone, but is less soluble on the acid and alkaline sides of this point. Precipitates obtained by small adjustment of the pH of acetone solutions of the isoelectric precipitate proved to be very little different in activity from the main fraction.

Chromatographic separation

Paper chromatograms. A 1 mg./ml. solution of marcescin was applied as a narrow band near to one end of a sheet of Whatman No. 1 filter-paper; after drying it was cut up into strips which were treated with solvents by capillary ascent in a closed water-saturated atmosphere. The bands were made visible by holding the strips in ammonia vapour for a short time, painting with a 0·1% solution of bromophenol blue and washing out the excess of reagent by immersing in two or three changes of water.

When dilute acids travel along filter-paper the acid usually does not extend as far as the water. These fronts are called the 'acid front' and the 'solvent front' respectively. Hydrochloric acid as weak as 0·05 N moved the antibiotic

completely to form a sharp band at the acid front. Phosphoric acid did likewise down to 0·2 %, but at 0·08 % the band was wide and trailing with no visible separation; 0·06 M-KH_2PO_4 was not sufficiently acid to move the antibiotic from its starting-point, but mixtures with from 3 to 0·1 volumes of 1 % phosphoric acid gave a separation, one band going to the acid front and the other remaining in the original position. Mixtures of dilute phosphoric acid or of 1–2 % acetic acid with 5–10 % of ammonium sulphate gave better band separation. In general, aqueous solutions having a pH of about 3 and containing some salt were able to effect separation. The salt probably causes dissociation of a complex.

It is evident that in order to separate protein mixtures the acidity must be as low as possible to get the biggest advantage from the different degrees of ionization of the components, while still being high enough to produce some movement of the material. The results with saturated aqueous (83 %) *n*-butanol were disappointing. Mixed with hydrochloric acid up to 5 N or with toluene-sulphonic acid up to 4 %, the material moved as a whole. When the hydrochloric acid concentration was kept constant and the butanol varied between 50 and 70 %, the band grew more diffuse and travelled less as the butanol concentration increased. With the butanol at 75 % and the HCl varying from 0·25 to 1 N, the band became more diffuse as the acid became weaker, but no separation of bands was observed. In mixtures with caustic soda or aqueous organic bases no band movement occurred. Two per cent benzoic acid or 2 % citric acid in 83 % *n*-butanol were not sufficiently acid to move the band, but with 1 % acetic acid there was some movement, and with 0·1 % phosphoric acid or 1 % trichloroacetic acid there was some separation into two components.

No separation resulted from the use of acetone (10–80 %) methanol, ethanol, *iso*propanol or dioxane in combination with dilute acids.

Silica gel columns. In view of the indications of heterogeneity, silica gel columns were used in an attempt to isolate definite fractions. Acid-washed silica gel of 40–60 mesh was adjusted to pH 6 with a phosphate buffer. A 19 × 0·7 cm. column containing 7 g. silica gel adsorbed about 100 mg. of marcescin at pH 6. It was then washed successively with 0·2 M phosphate buffers of pH 5, 4 and 3, acids of increasing strength, first aqueous and then in 60 % acetone. Fractions were examined by adding ammonia to samples of effluent at frequent intervals and noting any precipitation, the eluent being changed when only a faint turbidity was produced.

Precipitable material started to come through at pH 5·8. Several fractions were collected which did not differ greatly in activity. They were combined to give four main ones, as shown in Table 5. In contrast to the starting material, however, they proved capable of a partial separation by isoelectric precipitation into a pigmented acidic fraction of low activity (under 1 million u./g.) and a highly active colourless basic fraction (8 million u./g.). The more acid the point of precipitation, the lower was the activity (Table 5).

A second similar column was eluted exhaustively with a pH 5 buffer, and then 0·5 N-HCl and a 60:40 mixture of acetone and N-HCl. In the third and

fourth colums the buffer was omitted. In these, nearly all the material was recovered by the aqueous eluents (Table 6).

Table 5. *Isoelectric precipitation of fractions from silica gel*

	A			B			C			D		
Fraction eluted at/by ...	pH 6·0–5·0			pH 5·0–3·0			Aqueous acid			Acid acetone		
Wt. (mg.) ...	11·7			10·8			17·0			21·4		
Insol. dil. acid (mg.) ...	—			Trace			4·3			—		
First opalescence ...	pH 6			pH 4			pH 3·5			pH 5·5		
Isoelectric fractionation ...	pH	Wt. (mg.)	Activity*	pH	Wt. (mg.)	Activity*	pH	Wt. (mg.)	Activity*	pH	Wt. (mg.)	Activity*
	7·5	2·3	5·5	5·2	1·9	0·6	4·3	6·1	0·4	6·6	3·0	1·5
	8·3	1·0	5·5	7·0	1·3	6	6·5	2·8	3	8·2	9·0	8
	8·8	3·8	8	7·4	2·8	5·5	9·2	2·1	8	9·0	7·5	8
	Alk.	1·0	8	Alk.	1·0	8	—	—	—	—	—	—

* Units $\times 10^6$/g.

Table 6. *Fractionation of marcescin on a silica gel column*

Starting material (mg.)	Soluble in pH 5 buffer: Pptd. pH 6·2 (mg.)	Soluble in pH 5 buffer: Pptd. pH 9 (mg.)	Soluble 0·5 N-HCl: Pptd. pH 6·2 (mg.)	Soluble 0·5 N-HCl: Pptd. pH 9 (mg.)	Soluble 60 % acid acetone: Pptd. pH 6·2 (mg.)	Soluble 60 % acid acetone: Pptd. pH 9 (mg.)
108	10·8	61	6·7	5·1	7·7	8·2
102	.	.	16·7	60	5·1	0·7
121	.	.	16·7	71·3		

The acidic fractions were removed by precipitation at pH 6·2, re-dissolved in acid and reprecipitated. They had an activity of less than 1 million u./g. The main fractions were precipitated at pH 9 and were active at a dilution of 1/8 million. The fraction taken off by acid acetone contained relatively more of the acid fraction than those by aqueous buffer or acid. Passage through the column affected the equilibrium of the mixture, probably by holding back some of the acid fraction and making the resulting mixture separable.

The activity of the acidic fractions was due to mixture with the basic substance, since the ratio of activities and toxicities was the same for the two fractions, and a small amount of the basic fraction could be recovered by reworking the acidic one. These two fractions corresponded to those separable on paper with 1 % acetic acid containing 7 % ammonium sulphate. The acidic fraction on such a chromatogram did not move in 18 hr., whereas the basic fraction in 2 hr. moved 4·5–5 in. leaving behind a very small fraction at the starting-point.

It was possible to get an imperfect separation by dissolving the original material in 1 % acetic acid and adding 7 % ammonium sulphate, which precipitated a pigmented fraction. Even after reprecipitation it had an activity of 2·5 million u./g. This illustrates that solvents which effect separation on a paper chromatogram give some separation when used in the ordinary way, particularly if one component does not move, since a substance will not move with a solvent unless it is soluble in it. By the carbazole and Molisch tests the pH 9 fraction contained no carbohydrate; it contained 0·03 % of P.

The pH 6·2 precipitate contained about 7 % of carbohydrate and 0·9 % of phosphorus, and was probably largely nucleoprotein. A preparation free from carbohydrate was also obtained without the use of silica gel by repeated precipitation at pH 9, and by repeated precipitation of the picrate followed by solution in 70 % acid acetone and precipitation with excess acetone.

Properties of marcescin

Marcescin is readily soluble in water at pH 3, but on gradual neutralization the solution becomes increasingly opalescent, and gives a flocculent precipitate at *c.* pH 7. Precipitation is maximal by pH 9 and is not diminished by more alkali. The solubility at pH 7 is about 0·5 mg./ml. and at pH 9 0·02 mg./ml.

Marcescin dialyses readily through a cellophan membrane from acid solution and less readily from a solution in ammonium citrate or from broth. No active substance passed through a collodion membrane into distilled water from a citrate culture centrifugate.

Marcescin contains 13·3 % of N. It is precipitated by picric acid, the picrate being soluble in 70 % acetone and convertible to an active hydrochloride. It is precipitated by trichloroacetic, toluenesulphonic and tannic acids, and by acid dyes, the compound with bromophenol blue being very sparingly soluble. Marcescin gives precipitates with nucleic acid, insulin and heparin. All this is in accord with its being a basic peptide.

The biological activity was not destroyed by incubation with commercial pepsin, commercial trypsin, or a mixture of peptidases extracted from pig intestine. In this it resembles the antibiotic polypeptide, bacitracin (Anker, Johnson, Goldberg & Melleney, 1948).

Marcescin has strong surface-tension lowering properties; solutions foam if shaken, and it is adsorbed on glass surfaces and many precipitates. Considerable loss occurred in filtering citrate or broth culture fluids through a gradocol membrane (Elford, 1931) of pore size 0·63 μ., but all the activity came through one of pore size 0·8 μ. An L 2 Berkefeld candle at pH 7 and Seitz filtration at pH 3 or 6 removed all activity. A small amount passed through a Seitz filter at pH 1·4 or 12.

Marcescin is insoluble in anhydrous ethanol, *iso*propanol, *n*-butanol, acetone, dioxane, pyridine, morpholine, diethylamine and glacial acetic acid, but it is soluble when the solvents are diluted with water, for example 90 % acetic acid, 90 % phenol, 50 % pyridine, 70 % *iso*propanol and 65 % acetone. It is precipitated from the last by addition of ammonia. Its adsorption spectrum, similar to that of a histone, showed a small hump at 260 mμ. (possibly due to arginine) and intense adsorption at 240 mμ. due to amino-acids.

Marcescin is stable to boiling for at least 3·5 hr. at any pH between 2 and 12. The activity of a 1 mg./ml. aqueous solution at pH 2, or of a 1 mg./ml. suspension at pH 7 was unchanged after 8 weeks at room temperature. A solution of 0·01 mg./ml. at pH 7, however, lost 80 % of its activity in that time. There was no loss from a 0·01 mg./ml. solution in 3 % ammonium citrate in 8 weeks. This and other evidence suggests that marcescin may form a complex with the

citrate ion. Sterile citrate culture fluids were stable for several days at pH 3 or 6, but not at pH 9. Thus it is more stable in acid than in slightly alkaline solution.

ANTIBACTERIAL PROPERTIES OF MARCESCIN

Marcescin has a powerful action on *Corynebacterium diphtheriae* (the growth of which it inhibits at a dilution of 1/100 millions), *Pasteurella septica*, *Clostridium welchii* and *Staphylococcus aureus*. It inhibited the stock testing strain of *Staph. aureus* at 1/5 million dilution, and two others were more sensitive (up to 1/12 million). It is less active against *Mycobacterium tuberculosis* and *Streptococcus* spp. and still less active against *Shigella* spp. and *Escherichia coli*. The titres listed in Table 7 were determined in digest broth except for *Mycobacterium tuberculosis* which was tested in Dubos's medium (Dubos & Davis, 1946).

Table 7. *Activity of marcescin against various pathogenic bacteria*

	Inhibitory concentration (μg./ml.)
Corynebacterium diphtheriae	<0·01
Pasteurella septica	0·02
Clostridium welchii	0·03
Staphylococcus aureus (3 strains)	0·08–0·2
Mycobacterium tuberculosis	0·7
Haemolytic streptococcus	0·8 – 2·0
Streptococcus faecalis	0·8 – 2·0
Shigella flexneri	2·0
Escherichia coli	3·2

Effect of conditions of test on activity

Although it was difficult to obtain reproducible titres owing to the appearance in the test cultures of resistant organisms, it was not possible to vary activity much by purposeful alterations in the conditions of the titration. There was, for example, little difference in the activity when tested in broth at pH values from 6 to 8.

The activity against *Escherichia coli* was not affected by the presence of complex nitrogen compounds, because the same titre was obtained in digest broth, and in a chemically defined medium of salts, glucose and asparagine. The activity against *Staphylococcus aureus* in broth was not affected by 1 % glucose or 5 % of a casein digest. In 5 % horse serum broth, however, staphylococci were five times as sensitive to marcescin as in nutrient broth. The antibiotic was unaffected by thiolacetic acid or cysteine.

Concentrations of marcescin less than that necessary to inhibit growth completely, lengthened the lag, i.e. the time before growth was apparent to the naked eye. Thus, 0·01 u./ml. inhibited growth for 7 hr., and 0·1 u./ml. for 12 hr. Young (5 hr.) cultures seemed equally susceptible to marcescin as older (22 hr.) cultures.

The size of inoculum affected the sensitivity of staphylococci. An inoculum of a 1/2000 dilution of an overnight culture needed four times, and one of

1/20,000 needed twice, the concentration of marcescin which inhibited the growth of an inoculum of a 1/200,000 dilution.

With staphylococci on a cylinder plate, the area of growth inhibition was surrounded by a narrow zone having more intense reducing properties, as shown by methylene blue, than the rest of the culture. This is similar to the zone described by Dufrenoy & Pratt (1947) on penicillin plates.

Marcescin had little effect on the morphology of bacteria, except to diminish the size of the cells somewhat and in Gram-positive forms to produce a sticky or granular growth that stained Gram-negative.

Adsorption by bacteria

Staphylococci. Marcescin is strongly adsorbed by bacteria. A washed culture of *Staph. aureus* of opacity 4 (Brown's opacity tubes, Burroughs Wellcome Co.) was shaken for 2 hr. at 37° with varying concentrations of marcescin in pH 7 phosphate buffer. The bacteria were spun down and the supernatant assayed (Table 8). A loopful of the spun organisms was seeded into 5 ml. of fresh broth. Only those which had been treated with the lowest concentration (20 u./ml.) grew out in 24 hr. The clear broth of the seeded cultures was not inhibitory—much of the antibiotic was still adhering to the organism and preventing growth. Thus it is impossible to say whether a large concentration of the antibiotic is bacteriostatic or bactericidal, since the cells seeded into the testing broth carry firmly held antibiotic with them. In broth, the adsorption by broth-grown cultures is slightly less complete. A culture of staphylococci shaken with 400 u./ml. left 30 u./ml. in the supernatant. Staphylococci grown in broth adsorbed rather less readily than those grown in a chemically defined medium.

Table 8. *Adsorption of marcescin from phosphate buffer* (pH 7) *by staphylococci*

Marcescin added (u./ml.)	Marcescin in supernatant (u./ml.)	Growth (24 hr.) on explanting organisms
2000	1600	–
1000	600	–
200	4	–
20	<2	+

Other bacteria. Different species of bacteria adsorbed marcescin to about the same extent. Washed organisms suspended in pH 7 buffer containing 100 u./ml. and equal in opacity to an overnight culture diminished the concentration in the supernatants to below 2 u./ml. whether they were staphylococci (sensitive, Gram-positive), *Pasteurella septica* (sensitive, Gram-negative) or *Escherichia coli* (fairly resistant, Gram-negative). In another experiment both staphylococci and *Esch. coli* at about 10 times the concentration of an overnight culture diminished 400 u./ml. to less than 2 u./ml., and 2000 to 160 u./ml., in the supernatant after adsorption. Thus the different sensitivities of bacteria to marcescin did not appear to be due to differences in the degree of adsorption of the substance, nor was it likely that the basic marcescin was being specifically adsorbed on the ribonucleic acid of Gram-positive bacteria.

To decide whether marcescin did in fact kill bacteria, a substance was sought which would combine with it and neutralize its activity. Both bromophenol blue and heparin combine with marcescin but neither was able to reverse the inhibition to any extent.

Bromophenol blue itself did not inhibit the growth of staphylococci up to concentrations of 0·1 %. When 0·025 % of bromophenol blue was mixed with decreasing concentrations of marcescin in broth, which was then seeded with staphylococci, there was growth only at about 1 u./ml. and none at 3 u./ml., showing that bromophenol blue did not neutralize the activity of marcescin to any great extent. Heparin precipitates marcescin when both are at a concentration of 0·1 mg./ml. and causes some diminution in the antibacterial activity. Thus addition of an amount of heparin from 10 to 1000 times that of the marcescin used diminished the activity of marcescin to 25 %, but failed to enable staphylococci to grow after treatment with large concentrations of the antibiotic.

Serratia marcescens. In view of the strong adsorption of marcescin by bacterial cells it was important to see to what extent *S. marcescens* adsorbed it, and what effect this had on the titres of culture fluids. *S. marcescens* cells were washed off agar slopes (1 slope/ml.) into broth, water 3 % ammonium citrate solution, each containing 150 u./ml. of marcescin. After 30 min. at 37° the organisms were spun down and the supernatant assayed. Table 9 gives the results of two experiments and shows that citrate prevented the adsorption; this may explain the efficacy of citrate media in producing the antibiotic.

The bacterial cells in Exp. 2 were extracted with 3 volumes of acetone, spun down and then re-extracted with 0·05 N-HCl in 75 % acetone. The extracts were evaporated to dryness, leached with water and the aqueous extract spun clear. This contained about 60 units, or more than half of the adsorbed antibiotic. It appears that citrate media, combined with the acid acetone method of extraction reasonably safeguarded against losses due to adsorption on the bacteria.

Table 9. *Adsorption of marcescin by* Serratia marcescens

	Units added	Units in supernatant from		
		Water	Broth	3 % citrate
Exp. 1	120	20	7	•
Exp. 2	120	8	16	128

Table 10. *Adsorption of marcescin by* Serratia marcescens *from water*

	Culture grown at			
	24°		37°	
	In citrate	In broth	In citrate	In broth
Units added	300	300	300	300
Units in supernatant	7	12	26	26
Units extracted from cells by 75 % acid acetone	120	80	400	80

Cells grown in citrate medium or in broth adsorbed marcescin at 37° or at 24° to the same extent. The averages of three experiments are shown in Table 10.

Resistant forms. After eight to ten subcultures, some of several days' duration, *Staphylococcus aureus* could grow in 1 u./ml. of broth and *Pasteurella septica* in 0·5 u./ml. This small amount of acquired resistance was lost at the first subculture in absence of the antibiotic. 'Adapted' organisms tend to grow in tight pellets or strings at the bottom of the tube.

Action on enzyme systems. Marcescin inhibited the reduction of methylene blue by staphylococci in broth even in the presence of succinate, lactate, acetate, aspartate and fumarate. A concentration of 10–50 u./ml. partially, and 100–500 u./ml. totally, inhibited the enzyme systems involved. Marcescin also inhibited the reduction by *Staphylococcus aureus* of nitrate to nitrite. Five u./ml. diminished the reduction of nitrate to about 20 % of the control, and 15 u./ml. halved the rate of methylene-blue reduction in presence of succinate.

The effects were used as a means of quickly assaying culture fluids to judge when maximum titre had been reached. The amount of inhibition of enzyme action was not always proportional to the unitage measured by inhibition of growth; the tests were therefore abandoned as a means of assaying culture fluids.

Toxicity. Marcescin is very toxic, with an M.L.D. for mice orally or intravenously of under 62 mg./kg. The LD50 for mice was 125 mg./kg. by subcutaneous injection, and the M.L.D. by the intraperitoneal route was 2·5 mg/kg. Soon after injection the animals became very excited for a brief period, then lethargic, and just before death a series of rapid tremors ran from the head over the dorsal surface. In animals dying from intraperitoneal or intravenous injection there was acute abdominal haemorrhage and engorged auricles and right ventricle. Injection of doses of 1–5 mg. into the shaved skin of rabbits appeared to inhibit the infection by *Staph. aureus* injected at the same time, but also caused severe necrosis.

Action on blood cells. Horse blood was haemolysed immediately by a concentration of 1·25 mg./ml., and in 2 hr. by 0·005 mg./ml.

The action on leucocytes was determined by the method of Abraham, Chain, Fletcher, Gardner, Heatley, Jennings & Florey (1941); direct observation under dark ground illumination. A concentration of 1·25–2·5 mg./100 ml. caused loss of motility of about 50 % of cells in 1 hr.; the corresponding figure for penicillin is 500–1000 mg./100 ml. The effect on phagocytosis was less than would be expected from the above figures. In concentrations of 40 and 4 mg./100 ml. leucocytes engulfed up to half the number of bacteria that were taken up by the control leucocytes. At 0·4 mg./100 ml. there was no inhibition of phagocytosis. In these experiments the bacteria were added immediately after the drug.

Therapeutic properties. Three doses of 1 mg. subcutaneously at 0, 14 and 28 hr. after infection failed to protect mice against 100 M.L.D. of *Staph. aureus* ('Burroughs Wellcome mouse virulent strain') administered with mucin, or against 5 lethal doses of *Staph. aureus*. Similar subcutaneous doses failed to protect mice against 100–1000 M.L.D. of a mouse-virulent group A haemolytic

streptococcus, 'Richards', given intraperitoneally. Three doses of 0·5 mg. subcutaneously at 0, 14 and 25 hr., or 5 doses of 0·2 mg. at 0, 6, 14, 20 and 25 hr., failed to protect mice against 100 M.L.D. of *Pasteurella septica* (N.C.T.C. 2848).

DISCUSSION

It is not certain whether the activity of culture filtrates reported by Eisler & Jacobsohn (1935) and by Lichstein & van der Sand (1946) was due to marcescin. All the active substances were apparently colourless and thermostable and their antibacterial spectra are similar. Eisler found activity in his cultures of *Serratia marcescens* at 2–24 days at 22–26° or 1–4 days at 37°. Lichstein's substance was not present at 18 hr. but appeared in 3-week-old cultures. Our antibiotic appears to differ from these. It is not produced at 37°, and is present in maximum yield at 18 hr. at 24°, when using large seedings and vigorous aeration. Under less favourable conditions of growth it may be present for as long as 8 days, but it disappears from broth culture and would not survive for 3 weeks.

In our experience there was little connexion between the amounts of pigment and antibiotic formed except that both tended to be present during rapid growth. Pigment formation was poor and transitory in chemically defined media, and marcescin was produced equally well by the pigmented and achromogenic variants of our culture. In its resistance to digestion by proteolytic enzymes it is similar to other polypeptide antibiotics, e.g. bacitracin (Anker *et al.* 1948).

The activity of marcescin does not appear to depend on the presence of the haemorrhagic polysaccharide of Hartwell *et al.* (1943). Our purest product contained only traces of carbohydrate (probably impurity), and whereas marcescin is stable for several hours at 100° or in N-HCl for 6 hr. at 100°, the polysaccharide of Hartwell *et al.* was rapidly destroyed at 100° or in 2N-HCl at 37°.

Marcescin is difficult to free from acidic impurities; when converting the picrate to hydrochloride the process must be repeated several times to get a product free from picric acid. The trace of carbohydrate and phosphorus in our purest product is probably due to adsorbed nucleoprotein.

The most characteristic feature in the antibiotic action of marcescin is the ease with which it is adsorbed by bacteria. Staphylococci are able to remove 98 % of a high concentration of marcescin from solution. Owing to this it is not possible to say whether high concentrations of marcescin are bactericidal or bacteriostatic, since enough is carried over in the inoculum to continue to inhibit growth. The so-called bactericidal action of other antibiotics may be due to a similar phenomenon. The strong adsorption of the antibiotic by bacteria may account in part for its activity at high dilutions, and for the need for higher concentrations to inhibit larger implants of bacteria.

REFERENCES

Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A. & Florey, H. W. (1941). Further observations on penicillin. *Lancet*, ii, 182.

Anker, H. S., Johnson, B. A., Goldberg, J. & Melleney, F. L. (1948). Bacitracin: methods of production, concentration and partial purification, with a summary of the chemical properties of crude Bacitracin. *J. Bact.* **55**, 249.

Bergey, D. H. (1948). *Manual of determinative bacteriology*, ed. Breed, R. S., Murray, E. G. D. & Hitchins, A. P., 6th ed. London: Baillière, Tindall and Cox.

Breed, R. S. & Breed, M. E. (1926). *Serratia indica* Bergey, the red chromogenic bacterium originally isolated by Koch in India. *J. Bact.* **11**, 76.

Dubos, R. J. & Davis, B. D. (1946). Factors affecting the growth of tubercle bacilli in liquid media. *J. exp. Med.* **83**, 10.

Dufrenoy, J. & Pratt, R. (1947). Cytochemical mechanisms of penicillin action. I. Oxidation-reduction levels. *J. Bact.* **53**, 637.

Eisler, M. & Jacobsohn, I. (1935). Über die antagonistisch Wirkung steriler Bouillonextracte aus *Bacterium prodigiosus*. *Z. Hyg. InfektKr.* **117**, 76.

Elford, W. J. (1931). A new series of collodion membranes suitable for general bacteriological use, especially in filterable virus studies. *J. Path. Bact.* **34**, 505.

Hartwell, J. L., Shear, M. J. & Adams, J. R. (1943). Nature of the haemorrhage-producing fraction from *Serratia marcescens* culture filtrate. *J. Nat. Cancer Inst.* **4**, 107.

Hettche, H. O. (1932). Untersuchungen über die bakteriziden und anthrakoziden Bestandteil von *Bacillus pyocyaneus* und *Bacillus prodigiosus*. *Arch. Hyg., Berl.*, **107**, 337.

Lichstein, H. C. & van der Sand, V. (1946). The antibiotic activity of Violacein, Prodigiosin and Phthiacol. *J. Bact.* **52**, 145.

Marchal, J. G. & Girard, T. (1947). Phénomenès d'oxydoréduction mis en evidence avec le molybdate d'ammoniaque ajouté dans les cultures bactériennes. *C.R. Soc. Biol., Paris*, **141**, 118.

Pederson, C. S. & Breed, R. S. (1928). The fermentation of glucose by organisms of the genus *Serratia*. *J. Bact.* **16**, 163.

Reed, G. B. (1937). Independent variation of several characteristics of *S. marcescens*. *J. Bact.* **34**, 255.

(*Received* 4 *January* 1950)

Red-Leg in Tree-Frogs Caused by *Bacterium alkaligenes*

By ELLEN M. MILES

National Institute for Medical Research, Mill Hill, London, N.W. 7

SUMMARY: The individual animals in a large batch of European tree-frogs imported into England from Italy were found to be suffering from what proved to be, with two exceptions, a uniformly fatal attack of typical red-leg. *Bacterium alkaligenes* was isolated in profusion in pure culture from the heart blood and lymph fluid of all the frogs examined. The strain was highly susceptible to streptomycin, and the only animals to recover were two that were treated with 1000 units of the antibiotic, in divided doses over 9 days.

Red-leg in frogs has in the past always proved to have been due to a bacteraemic infection by *Proteus hydrophilus* Bergey *et al.* (see Kulp & Borden, 1942) or to very similar bacilli. This report concerns red-leg due to an entirely unrelated bacterium.

Red-leg in tree-frogs. In July 1949, during a search for fresh *Pr. hydrophilus* strains, I examined a batch of over fifty European adult tree-frogs (*Hyla arborea* (Linn.)) which had arrived at the London Zoological Society's Gardens from North Italy, packed in a very crowded container. Soon after arrival, many deaths from red-leg occurred, and it was evident that the whole batch was likely to be attacked. Of the surviving frogs, some four-fifths died before examination was possible, but six were observed while dying, and examined immediately.

Their normally bright, moist green skins were dull and brownish. There were red pustules and a red gelatinous material round the mouth and on the fingers and toes. Bright red mottling and small petechial haemorrhages were present on the skin of the thighs and abdomen. The subcutaneous lymph was copious and blood-stained, and the body-cavity filled with blood-stained exudate. The heart was pale, with prominent superficial vessels. The lungs were intensely bloody in three animals, and apparently normal in the rest. The liver was greenish and soft, and the surface of intestines flecked with sub-peritoneal haemorrhages. The kidney and spleen were apparently normal. Gram-negative bacilli were in profusion in the lymph and peritoneal exudate; none were seen in the heart blood. Direct plating of lymph and heart blood in all six cases yielded profuse, pure cultures of a Gram-negative bacillus which proved to be typical strains of *Bacterium alkaligenes.*

Description of the strain isolated. A Gram-negative non-acid fast rod, $0{\cdot}5 \times 1$–$1{\cdot}5\,\mu$., feebly motile by peritrichate flagella. Mainly coccobacillary, occasionally filamentous. On horse blood agar, in one day at 37°, the colonies were 0·5 mm., round, moist, domed, semi-translucent and yellowish; non-haemolytic. Failed to ferment a wide range of the bacteriological 'sugars'. Citrate, indole, methyl red, Voges-Proskauer, catalase, NH_3 and H_2S negative. Nitrate not reduced to nitrite. Gelatin not liquefied. Litmus milk feebly alkaline in 2 days at 37°, without ropiness. Grows better at 37° than at 20°.

Not agglutinated by antisera to authentic strains of *Haemophilus bronchisepticus*.

Three normal adult specimens of *Rana temporaria* were given 0·4 ml. of an 18 hr. broth culture subcutaneously. All survived for 4 weeks without a sign of disease, and displayed no abnormalities at post-mortem examination. No specimens of the tree-frog were available for pathogenicity tests.

Treatment of the red-leg by streptomycin. Epizootics of red-leg may seriously diminish or wholly destroy colonies of frogs in captivity. Suppression of the epizootic by segregation and chilling of the individual frogs, as recommended by Emerson & Norris (1905) though successful, is laborious, and may spoil the animals, for example, as objects of physiological research. The organism was susceptible to streptomycin *in vitro*. Titrated in heart-broth in a two-fold dilution series, streptomycin was bacteriostatic for the *Bact. alkaligenes* strain in half the minimum concentration needed for bacteriostasis of Waksman's susceptible strain of *Bact. coli*.

The curative effect of streptomycin was therefore tested. From the remnant of the original batch five tree-frogs were selected and each of them was isolated. Three received subcutaneous injections of 0·2 ml. of a 1000 unit/ml. solution of streptomycin in distilled water; the dose was repeated on the 2nd, 3rd, 6th and 9th days, a total of 1000 units being given. One of these animals had advanced red-leg, of a degree which had in other animals indicated death in 1–2 days; it survived for 1 week. Two with incipient red-leg—sluggish, with dull, dark skins and faint pink flush over the thighs and belly wall—were bright and active in 48 hr., and recovering their glossy green colour. They recovered completely, and were well 3 months later.

The rest of the batch of tree-frogs in a sense serves as a control in this test, because all without exception died. The two remaining frogs of the five selected had no visible signs of red-leg at the beginning of the streptomycin test. They received only distilled water in 0·2 ml. doses; both died of red-leg, one in 2, the other in 4 days.

COMMENT

Though its power to reproduce the disease in the susceptible animal could not be proved, there can be little doubt that the *Bact. alkaligenes* was the cause of the haemorrhagic septicaemia in these tree-frogs. It must therefore be added to *Proteus hydrophilus* as one of the causes of red-leg in amphibians. Its non-pathogenicity for common frogs (*Rana temporaria*), which were from a batch known to be susceptible to experimental *Proteus hydrophilus* infection, suggests that its host range is more restricted than that of *Pr. hydrophilus*.

The very limited evidence from the therapeutic tests suggests that streptomycin treatment is well worth a trial in red-leg of captive frogs caused by this bacillus.

I am indebted to Dr E. Hindle, the Scientific Director of the Zoological Society of London, for the opportunity to investigate the epizootic of red-leg; and to Mr H. Proom of the Wellcome Research Laboratories for tests with *H. bronchisepticus* antisera.

REFERENCES

Emerson, H. & Norris, C. (1905). 'Red Leg', an infectious disease of frogs. *J. exp. Med.* **7**, 32.

Kulp, W. L. & Borden, D. G. (1942). Further studies on *Proteus hydrophilus*, the ecological agent in 'Red Leg' disease of frogs. *J. Bact.* **44**, 673.

(*Received* 15 *January* 1950)

Factors Influencing the Early Phases of Growth of *Aerobacter aerogenes*

By S. DAGLEY, E. A. DAWES and G. A. MORRISON

Department of Biochemistry, School of Medicine, Leeds

SUMMARY: The method used in maintaining cultures of *Aerobacter aerogenes* affects the relationship between duration of lag phase and age of inoculum in a glucose ammonium salt medium. Organisms kept in broth may show pronounced early lag whilst those from agar slopes do not. Previous subcultivation with aeration decreases the lag. Bacteria showing no lag may do so when they have been washed; glutamate, α-ketoglutarate and succinate partially remove the lag so produced, whilst malate, fumarate and aspartate do not.

The initial growth rate of light inocula showing no lag is increased by the addition of glutamate, aspartate, α-ketoglutarate, succinate and oxalacetate; but pyruvate, fumarate and malate have no action or are slightly inhibitory. The effect of these compounds over a concentration range of 0 to $1{\cdot}6 \times 10^{-3}$ g. mol./l. was studied quantitatively. Filtrates from growing cultures have a similar effect on initial growth rate; those taken during the late logarithmic phase are more effective than those taken earlier. The effects of both filtrates and added compounds are confined to initial growth; the rate of growth in the later phase, when turbidity becomes visible, remains unaltered. This behaviour is dependent on the amount of O_2 available in the medium.

Early lag is a phase of greatly reduced rate of growth rather than complete absence of cell division. Glutamate and other effective compounds increase the rate to values approaching normal.

Lodge & Hinshelwood (1943*b*) showed that when a glucose ammonium salt medium was inoculated with bacteria grown in a similar medium, the lag period which developed depended on the 'age' of the inoculating cells. When the latter were transferred in the early logarithmic phase there was a considerable lag ('early lag') which was attributed to the diffusion from the cells of metabolic intermediates necessary to allow rapid cell division to begin. Sterile filtrates from fully grown cultures were able, for example, to remove this lag because they supplied the cells with relatively high concentrations of these postulated intermediates. Kinetic studies of bacterial growth provide a method of investigating the reactions which occur before cell division begins. When growth responses similar to those obtained by filtrate additions are produced by known compounds, information may be furnished about the nature of the metabolites normally present during the early growth phases. When a culture ages it shows 'late lag' when inoculated into fresh medium. Morrison & Hinshelwood (1949) showed with *Escherichia coli* that such lag was partially abolished by additions of glutamate, aspartate and other compounds. Whereas diffusion of growth intermediates from the cells may account for early lag, several additional factors undoubtedly determine late lag (Lodge & Hinshelwood, 1943*b*). Our interest has been the possibility of studying growth intermediates, and we have investigated the early phases of growth.

The work of Lwoff & Monod (1947) with *Esch. coli* and of Ajl & Werkman

(1948) with *Esch. coli* and *Aerobacter aerogenes*, concerning compounds which can partially reverse the retardation of growth due to CO_2 deficiency, has also established the importance of glutamic and aspartic acids and closely related non-nitrogenous compounds in the metabolism of these organisms. It was of interest to study the response to these and other compounds when the carbon dioxide tension was adequate for normal growth. It has already been reported (Dagley, Dawes & Morrison, 1949*a*) that under these conditions the lag period produced by the addition of phenol to the culture is largely removed by glutamic and succinic acids.

A study of the literature referred to above reveals that early lag was not observed for inocula of the size (or 'age') for which lag would be expected from the work of Lodge & Hinshelwood (1943*b*), although Lwoff & Monod (1947) found that cells which would grow without lag in test-tubes exhibited prolonged lag in flasks aerated by agitation at normal CO_2 tensions. We have observed large differences in lag, with different strains of the same organism but the same size of inoculum, and we have, therefore, studied the influence on lag of various conditions of culture before and during growth in the medium under investigation.

EXPERIMENTAL METHODS

The organism used throughout this work was *Aerobacter aerogenes*, N.C.T.C. 418. It was maintained by monthly serial subculture in two ways: (1) on nutrient agar, and (2) in nutrient broth. Growth curves were obtained as follows. Samples of the growing culture (*c.* 2 ml.) were withdrawn by Pasteur pipette at various times and killed by the addition of 2 drops of formalin. The bacterial population was determined turbidimetrically using microcells in a Spekker photoelectric absorptiometer with Ilford filters (neutral H 508 and blue no. 6). A relation between turbidity and total count (haemocytometer) was established. When details of the early stages of growth were required before the culture was turbid, viable counts were made by plating out 0·1 ml. of a suitable dilution on nutrient agar. Cultures were grown in Pyrex boiling tubes (6 in. $\times \frac{5}{8}$ in.), carefully cleaned by boiling with nitric acid followed by washing with glass-distilled water. Cultures were incubated at $39 \pm 0{\cdot}1°$. The adverse effect of traces of heavy metals and other impurities on bacterial growth in simple chemically defined media has been stressed by Poole & Hinshelwood (1940), Lodge & Hinshelwood (1939) and by Monod (1942). Reproducibility in quantitative experiments is impossible without these special precautions.

The basal medium contained: 5·4 g. KH_2PO_4; 1·2 g. $(NH_4)_2SO_4$; 12 g. glucose and 0·4 g. $MgSO_4.7H_2O$/l. of glass-distilled water, pH adjusted to 7·1. The salt solution and the glucose solution (3 % w/v) were sterilized separately and mixed on cooling. Portions of medium (25 ml.) were dispensed aseptically into sterile boiling tubes. Bacteria for any series of experiments were prepared by three serial subcultures in the basal medium and 0·1 ml. of the third culture at the phase of growth under investigation was used as inoculum. Thus the number of cells inoculated, as well as their age, varied.

Measurement of lag

For an actively-dividing culture the plot of the logarithm of bacterial population against time is linear and if there is no lag the growth curve will extrapolate to the logarithm of cells inoculated at zero time. If a lag takes place it may be defined as the intercept on a line drawn through the latter value parallel to the time axis. It is still convenient to use this definition even in cases when the transition from lag phase to logarithmic phase is not sharp (Hinshelwood, 1946). In several of our experiments, however, it was not necessary to resort to extrapolation since the lag period was followed directly by viable counts.

To ensure reproducibility of results when the effects of a series of factors affecting growth were compared, media were always inoculated from the same culture at the same time and with the same pipette; controls were run at least in duplicate. Agreement was not considered satisfactory if the times for the controls to reach a certain turbidity did not agree to within 5 min.

RESULTS

Factors influencing relationship between lag and age of inocula

The age of a growing culture has been defined arbitrarily as the time taken to reach its present population, starting from a haemocytometer count of unity ($1{\cdot}25 \times 10^{-6}$ cells/ml.; Lodge & Hinshelwood, 1943*b*). This age refers to the parent culture and the initial population of a daughter culture inoculated from it was 1/250 of the population of the parent, at that age, in our experiments. The present work was undertaken because we found that inocula derived from a strain maintained on nutrient agar slopes did not show lags of the duration expected from the relationship between lag and age so defined, which Lodge & Hinshelwood (1943*b*) established for another strain of *Aerobacter aerogenes*. There were, however, differences in technique since these workers maintained the organism by monthly subculture in broth, and carried out their experiments in media through which a gentle stream of sterile air was passed. Accordingly, we maintained our strain in this way as well as on slopes and constructed lag-age curves for the strains kept in both ways. Whichever strain was studied it was previously subcultured serially in the basal medium three times before inoculation. The results are summarized in Fig. 1; in unaerated cultures the strain maintained on slopes showed no lag over the range of age investigated (curve *D*), whilst that maintained in broth (curve *A*) showed pronounced early lag with a minimum in the curve about one-third of the way through the logarithmic phase. For curve *A* the maximum lag at 350 min. occurred towards the end of the logarithmic phase and after this point, when the cells were no longer dividing, the lag fell sharply. Fig. 1 was constructed by following the growth curves of the daughter cultures turbidimetrically, but the sharp rise in lag at ages of inoculum between 200 and 300 min. in curve *A* was also confirmed by viable counts for an inoculum taken at 220 min.; it was found that a lag of about 240 min. occurred which was largely removed by addition of glutamate.

Curves *B* and *C* were both obtained with the organism stored in broth and in the preliminary three passages through basal medium the cultures were aerated at normal CO_2 tension in the manner adopted by Lodge & Hinshelwood (1943*b*). In case *B*, such cells were tested for lag in an aerated medium and, in case *C*, in an unaerated medium. When cells to be grown in conditions of full aeration are not previously trained to these conditions a considerable lag develops.

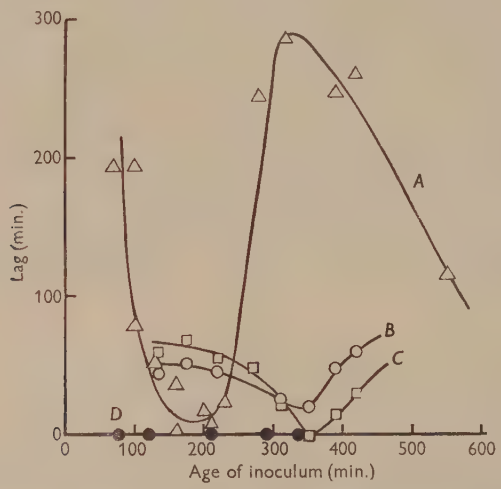


Fig. 1. Effect of method of maintenance of strain on lag-age relationship. Strain maintained: (*A*) in broth △, inocula grown without aeration, tested without aeration; (*B*) in broth ○, inocula grown with aeration, tested with aeration; (*C*) in broth □, inocula grown with aeration, tested without aeration; (*D*) on slopes ●, inocula grown without aeration; tested without aeration.

Lwoff & Monod (1947) observed that cells inoculated into media aerated by agitation in a flask showed lag, and for this reason they added Na_2S which acted as a 'facteur de départ' and removed the lag. In our experiments, however, we found that once the bacteria had overcome this lag period in an aerated medium, and were in the logarithmic phase, they were able to grow without abnormal lag in a new aerated medium containing no additional substance. A comparison of curves *A* and *C* shows that the cells which were not trained to aeration were also distinguished from those which were so trained by their behaviour in unaerated media.

Effect of various compounds on early phases of growth

Using inocula of the organism maintained on slopes to give an initial population of *c.* 2×10^4 bacteria/ml., it was found that the addition of small amounts of certain compounds enabled the cultures to become turbid before the control did, although in the region of turbidity for which growth curves could be accurately constructed, the growth rates were identical (Fig. 2). The compounds added must have exerted their effect before the cultures became visibly

turbid. As previously indicated, bacteria stored on agar slopes did not give measurable early lag with this size of inoculum. Viable counts of two cultures, one containing no Na L-glutamate and the other 6×10^{-3} g. mol./l., showed that the growth rate for the first few generations was markedly increased by the

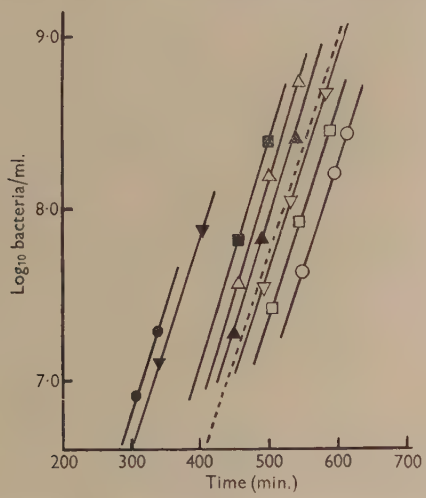


Fig. 2. Sections of growth curves determined by turbidimetric method. Cultures contained various organic acids at 0·06 g./l. Control – – – – –; acids: L-glutamic, ●; DL-aspartic, ▼; succinic, ■; oxalacetic, △; α-ketoglutaric, ▲; fumaric, ▽; pyruvic, □; malic, ○.

glutamate, but its effect persisted only to populations which were just visible, i.e. *c.* 5×10^6 cells/ml. Further, the growth curve for the control in Fig. 2 extrapolates to the initial population at zero time, so that in the first few generations growth in media containing glutamate, etc., must have been faster than in the control.

The concentration of all the compounds used in the experiments of Fig. 2 was 0·06 g./l. Similar sets of growth curves were obtained at five other concentrations using the same inoculum at the same time. From the six sets of results for each compound, the time displacement of each growth curve relative to that of the control was obtained (Fig. 3). Malate, fumarate and pyruvate were somewhat inhibitory in the initial stages of growth, whereas glutamate, aspartate, succinate and α-ketoglutarate promoted growth. The sample of oxalacetate was that used by Morrison & Hinshelwood (1949), prepared by the method of Fenton & Jones (1900) and the anomalous shape of the curve for this substance is accounted for by the presence of unoxidized malic acid which is inhibitory.

Lag shown by washed cells

Although the organism maintained on slopes showed no lag at ages at which other strains did so, lag was obtained by harvesting cells from a culture in basal medium and washing three times with phosphate buffer pH 7·1. Media

containing glutamate, succinate or α-ketoglutarate, when inoculated with cells so treated, became turbid much sooner than the control, but not when aspartate, citrate, acetate, fumarate or malate was added. As with Fig. 2, the growth curves were parallel in the region of visible turbidity, but unlike Fig. 2 extrapolation showed that the control culture had a lag period which was

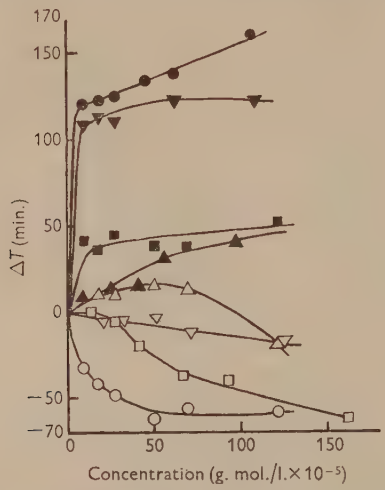


Fig. 3. Effect of concentration of various organic acids on initial growth. ΔT: displacement of growth curve (min.) with respect to control, positive values denote acceleration and negative values retardation of initial growth. Acids: L-glutamic, ●; DL-aspartic, ▼; succinic, ■; α-ketoglutaric, ▲; oxalacetic, △; fumaric, ▽; pyruvic, □; malic, ○.

decreased by adding glutamate, etc. These results were confirmed by viable counts. The effects produced by various added compounds on the lag shown by washed cells were similar to those described in the previous section, except that aspartate was effective in displacing the growth curve of unwashed cells but not in reducing the lag of washed cells.

The early lag phase

During the course of this work we obtained through the courtesy of Prof. Sir Cyril Hinshelwood a strain of *A. aerogenes* which, unlike the organism stored on agar slopes which we were using at the time, showed early lag but of much shorter duration than that with strain *A*, Fig. 1, later investigated. However, with small inocula giving an initial population of 300–400 bacteria/ml., cultures showed lags of 7 hr. or more. Growth over this period was followed by plate counts and the effect of additions of glutamate, succinate and α-ketoglutarate studied (Fig. 4). Growth rates were also followed at a later stage by turbidity measurements and were in each case the same as the control, although cultures with the additions present reached visible turbidity considerably earlier. Early lag is not a phase where cell division is totally suppressed, but one of greatly reduced growth rate, and the effect of additions of growth-

promoting substances is to increase the slower growth rate to a value approaching normal. This was confirmed in several other experiments using the viable count technique.

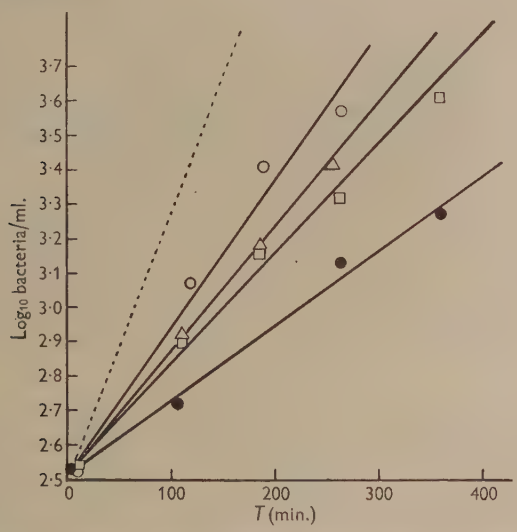


Fig. 4. Early lag of light inocula; effect of organic acids on decreased growth rate as followed by viable counts. Control, ●. Additions of 3×10^{-4} g. mol./l. of following acids: succinic, □; α-ketoglutaric, △; L-glutamic, ○. Dotted line indicates growth rate obtained by turbidimetric method when cultures had grown to populations $>10^7$ bacteria/ml. The organism used in these experiments was that obtained from Prof. Sir Cyril Hinshelwood.

Effect of inoculum size and culture filtrates on the early growth phase

Lodge & Hinshelwood (1943*b*) showed that sterile filtrates from fully-grown cultures abolished early lag. We observed that media to which such filtrates were added always reached visible turbidity before those with no additions, when a light inoculum was used. By the methods previously described this was shown to be due to the attainment of a higher initial growth rate in the former cases.

Inocula were taken from a growing culture at various times; samples of the same culture were also centrifuged, the clear supernatant boiled and 10 ml. additions made to 10 ml. lots of basal medium in which the inocula were to be tested. In this way it was possible to study not only the effect of filtrates of different ages on the initial mean generation time but also how the response obtained with various inocula depended on their age and size.

The results of such experiments (Fig. 5) show that (1) filtrates from the late logarithmic phase are more effective than those taken earlier in growth; (2) the response decreases with the age, i.e. with the size of the inoculum. It was also found that the response to glutamate and other compounds depended in a similar way on inoculum size. Although exact extrapolation of the curves in

Fig. 5 to the point $\Delta T = 0$ is difficult, it is possible to estimate that this occurs for ages between 410 and 450 min. Calculation shows that this corresponds to a parent log (cell population) value of 9·1 to 9·4, which in turn shows that filtrates will have no effect ($\Delta T = 0$), using this technique, when log (initial population) for the daughter culture is 6·7–7·0, i.e. when the initial population is 5 to 10×10^6 cells/ml. This is the point when the turbidity of the culture is sufficient to be measured by our methods, which is in accordance with our observations that filtrates and the various growth-promoting compounds exerted their effects earlier than this point, leaving the growth rate obtained from turbidities unaffected.

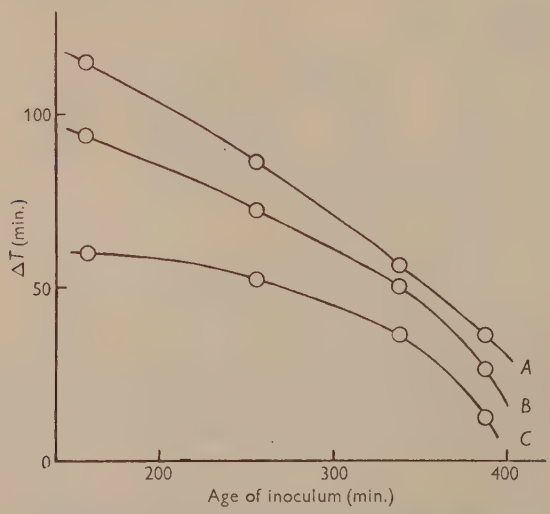


Fig. 5. Effect of culture filtrates and of inoculum size on initial growth. ΔT: displacement of growth curves with respect to control containing no filtrate addition. 10 ml. filtrate, 10 ml. basal medium. Filtrates from cultures of ages (*A*) 370 min., (*B*) 225 min., (*C*) 125 min.

It was observed by Lodge & Hinshelwood (1943*a*) that when glutamic acid and other amino-acids were the sole source of nitrogen, the culture population did not attain its maximum unless the media were aerated, although the initial growth rate might be greater than in unaerated glucose ammonium salt media. We found that in unaerated cultures with glutamate, succinate or α-keto-glutarate as the sole carbon sources, visible turbidity was reached more rapidly than with cultures containing molecular equivalents of glucose; but the rate of growth soon decreased in the former cases, and the final stationary populations were considerably less than with glucose, with which growth also differed in being logarithmic throughout. These observations, together with those previously recorded, suggest that the various factors promoting growth do not exert their maximum effects unless the O_2 tension is optimal. We found that when growing cultures contained 0·6 mg. methylene blue/l. no decolorizing of

the dye occurred until visible turbidity was reached, but after this point the rate of reduction was relatively rapid so that a culture of 8×10^7 cells/ml. decolorized the dye in less than 30 min. and 3×10^8 cells/ml. did so in 15 min. Colour returned when cell division ceased in the stationary phase. Two salient facts about the mid-logarithmic phase of cells grown under these conditions, therefore, emerge: (1) the bacteria deplete the medium of O_2; (2) experiments with filtrates prove that compounds are present which can stimulate faster growth when transferred to a medium not depleted of O_2. Accordingly, we studied the growth of two cultures, both in basal medium containing no additions but one culture was grown in an unaerated tube, while through the other a stream of air of normal CO_2 content was passed as soon as visible turbidity was reached. In the latter culture there was a small increase in growth rate from the moment aeration began, and growth continued at this rate to give a stationary population higher than in the unaerated culture. Lodge & Hinshelwood (1943*a*) found a mean generation time of 32 min. for *A. aerogenes* when fully aerated, whilst when unaerated it was 40 min.

DISCUSSION

In our experiments previous subcultivation ensured that over thirty generations of bacteria had been produced when growing with glucose as sole carbon source (apart from CO_2) prior to inoculation into the experimental media. The fact that glutamic and aspartic acids, and non-nitrogenous compounds related to them, were able in small amounts to accelerate the initial processes of growth in the basal medium, whether by abolishing lag or increasing growth rate, suggests that they take part in rate-controlling reactions. This is supported by the observation that filtrates from growing cultures produced responses similar to the compounds tested, under the same conditions. These conclusions accord with those of Morrison & Hinshelwood (1949), who studied the effects produced by various compounds on the lag which results when cells are allowed to age. They also obtained evidence that the rate-controlling step was the production of the carbon skeletons necessary for synthesis from the glucose, and that subsequent amination was relatively rapid; they assigned to glutamate a key role in terminating lag. Roine (1947), working with a strain of *Torulopsis utilis*, produced evidence for the view that glutamic acid is the primary amino-acid formed in yeast from a carbon chain and ammonia, and is of first importance in the bio-synthesis of amino-acids and proteins.

Our experiments showing the dependence of early lag on the method of maintaining the cultures whereby the synthetic abilities of the bacteria in a basal glucose ammonium salt medium are undoubtedly modified, are compatible with the view of Lodge & Hinshelwood (1943*b*) that the early lag phase is governed by the rate of synthesis of essential metabolites. Our studies of the lag produced by washing are also readily interpreted by assuming that washing removes essential intermediates from the cells, just as high dilution can give rise to early lag by a similar process.

We found that culture filtrates and various active compounds affected initial growth but not growth in the later logarithmic phase, and we suggest that they

are able to exert their maximum effect only when the O_2 tension in the medium is optimal. There is abundant evidence for considerable changes in redox potential during bacterial growth (Hewitt, 1936; O'Meara, McNally & Nelson, 1947) and optimal requirements may, in consequence, be assumed to vary with the phase in the growth process. Thus, we found that bacteria lagged considerably in fresh medium through which air was passed, although when aerated in mid-logarithmic growth, when experiments with methylene blue indicated oxygen depletion, growth was accelerated. In their experiments on the role of CO_2 in bacterial growth, Lwoff & Monod (1947) also observed the adverse effect of aeration on light inocula. They postulated that reactions concerned with the synthesis or metabolism of glutamic acid are related to a favourable cellular redox potential, the establishment of which is rendered difficult by excessive aeration. This difficulty was not met when they grew their cultures in tubes instead of agitated flasks; but we found (Dagley *et al.* 1949*a*) that Na_2S, added to flasks by Lwoff & Monod (1947) as 'corps réducteur "protecteur"', in order to remove lag, also promoted growth in tubes. Winslow, Walker & Sutermeister (1932) and Lodge & Hinshelwood (1943*a*) assumed that increased growth in aerated cultures was due to removal of toxic products by the air stream, but from our experiments showing the relationship of aeration to growth response it would appear that O_2 has a more positive function and ensures the maximum rate of utilization of essential metabolites by the cells.

Ajl & Werkman (1948) drew attention to the fact that compounds compensating for CO_2 deficiency are also constituents of the Krebs oxidation cycle or are their natural precursors. For several reasons, however, a view assigning to this cycle a significant role in bacterial metabolism must be treated with reserve. As Ajl & Werkman (1949) pointed out, several of these compounds replace CO_2 anaerobically, and it is generally considered that the tricarboxylic acid cycle is an aerobic mechanism by which various substances can be oxidized to CO_2 and H_2O to yield energy to the system. We also found that whereas some compounds in the cycle accelerated growth others did not; it is therefore difficult to see why the latter should be prevented from entering this cycle, if it exists in bacterial systems. Furthermore, we found (Dagley *et al.* 1949*b*) that fluoroacetate, which blocks this cycle (Buffa, Coxon, Liébecq & Peters, 1949) had no effect on the growth of *A. aerogenes* over a range of concentration at which iodoacetate was strongly bacteriostatic. It is well established that the action of the latter is entirely different from that of fluoroacetate, since it combines with —SH groups.

One of us (G.A.M.) wishes to acknowledge the award of an Imperial Chemical Industries Ltd. Research Fellowship.

REFERENCES

Ajl, S. J. & Werkman, C. H. (1948). Replacement of CO_2 in heterotrophic metabolism. *Arch. Biochem.* **19**, 483.

Ajl, S. J. & Werkman, C. H. (1949). Anaerobic replacement of carbon dioxide. *Proc. Soc. exp. Biol., N.Y.*, **70**, 522.

Buffa, P., Coxon, R. V., Liébecq, C. & Peters, R. A. (1949). Experiments on the tricarboxylic acid cycle in brain and kidney tissues. 1*st Internat. Congr. Biochem.* Abstr. No. 52/3, p. 570.

Dagley, S., Dawes, E. A. & Morrison, G. A. (1949*a*). Application of bacterial kinetics to biochemical problems. *Nature, Lond.*, **163**, 532.

Dagley, S., Dawes, E. A. & Morrison, G. A. (1949*b*). Influence of amino-acids, and compounds in the Krebs oxidation cycle, on 'early lag'. *Biochem. J.* **45**, xxvi.

Fenton, H. J. H. & Jones, H. O. (1900). The oxidation of organic acids in presence of ferrous iron. Part I. *J. chem. Soc.* **77**, 69.

Hewitt, L. F. (1936). *Oxidation reduction potentials in bacteriology and biochemistry*, 4th ed. London: London County Council.

Hinshelwood, C. N. (1946). *The chemical kinetics of the bacterial cell.* Oxford University Press.

Lodge, R. M. & Hinshelwood, C. N. (1939). Physicochemical aspects of bacterial growth. Part IV. Conditions determining stationary populations and growth rates of *Bact. Lactis Aerogenes* in synthetic media. *J. chem. Soc.* p. 1683.

Lodge, R. M. & Hinshelwood, C. N. (1943*a*). Physicochemical aspects of bacterial growth. Part VIII. Growth of *Bact. Lactis Aerogenes* in media containing ammonium sulphate or various amino acids. *J. chem. Soc.* p. 208.

Lodge, R. M. & Hinshelwood, C. N. (1943*b*). Physicochemical aspects of bacterial growth, Part IX. The lag phase of *Bact. Lactis Aerogenes.* *J. chem. Soc.* p. 213.

Lwoff, A. & Monod, J. (1947). Essai d'analyse du role de l'anhydride carbonique dans la croissance microbienne. *Ann. Inst. Pasteur*, **73**, 323.

Monod, J. (1942). *Recherches sur la croissance des cultures bactériennes.* Paris: Herman & Cie.

Morrison, G. A. & Hinshelwood, C. N. (1949). Nitrogen utilization and growth of coliform bacteria. Part III. Nitrogen utilization and lag phase. *J. chem. Soc.* p. 380.

O'Meara, R. A. Q., McNally, P. A. & Nelson, H. G. (1947). The intra-cellular mode of action of the sulphonamide derivatives. *Lancet*, ii, 747.

Poole, A. & Hinshelwood, C. N. (1940). Physicochemical aspects of bacterial growth. Part VI. The influence of toxic substances on growth rate, stationary population and fermentation reactions of *Bact. Lactis Aerogenes.* *J. chem. Soc.* p. 1565.

Roine, P. (1947). On the formation of primary amino acids in the protein synthesis in yeast. *Ann. Acad. Sci. Fennicae*, A, **11**, 26.

Winslow, C.-E. A., Walker, H. H. & Sutermeister, M. (1932). The influence of aeration and of sodium chloride upon the growth curve of bacteria in various media. *J. Bact.* **24**, 185.

(*Received* 17 *January* 1950)

An Improved Colony Illuminator

By W. T. MOORE and C. B. TAYLOR

Imperial Chemical Industries Ltd., Butterwick Research Laboratories, The Frythe, Welwyn, Hertfordshire

SUMMARY: A colony illuminator is described which incorporates side illumination by means of a small ring fluorescent tube. Inspection of the Petri dish is facilitated by the use of a detachable 5 in. diameter plastic industrial inspection lens.

For some years the need has been felt for a good colony illuminator, for colony counts or morphological studies. Recent developments in the manufacture of fluorescent tubes and of plastic inspection lenses have allowed us to design an illuminator which not only provides good lighting but relative freedom from eye strain when the instrument is used for long periods. In the past, most

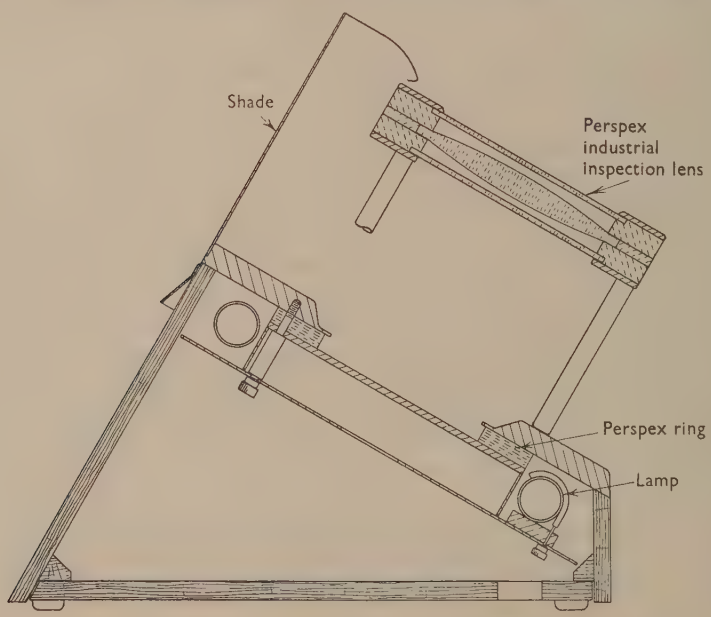


Fig. 1. Diagrammatic arrangement of colony illuminator ($\times \frac{1}{2}$).

counting chambers have employed diffused lighting through the base of the Petri dish, but Crisp (1940) described a chamber in which side lighting was used; a beam of light passed through a rectangular slot at the side of the Petri dish, and the colonies appeared in relief (by the shadows).

Our illuminator (Fig. 1 and Pl. 1) consists of a sloping box on which the Petri dish rests. A ring fluorescent lamp 8 in. diameter (Longlamps Ltd., Marshalsea

The improved colony illuminator

Road, London, S.E. 1), surrounds the dish and provides the illumination; a ring of Perspex placed between lamp and dish carries the light to the edge of the dish. The low temperature of the fluorescent lamp ensures that the chamber is not overheated. Further to this end the choke, which becomes warm after use, is housed in the three-pin plug.

A 5 in. diameter $\times 2$ plastic industrial inspection lens (Imperial Chemical Industries Ltd., Plastics Division) is supported over the dish on three legs. Two of the legs slide into sockets thus ensuring that the lens is rigidly held but easily removed. The lens is designed for use at a distance of 6 in. from the eyes and provides a substantially distortion-free image which can be observed with both eyes simultaneously. Ruled paper counting disks may be inserted under the dish if required.

REFERENCE

Crisp, A. E. (1940). A counting lamp for colonies of bacteria. *J. sci. Instrum.* **17**, 70.

(*Received* 18 *January* 1950)

Viability of Dried Bacterial Cultures

By MABEL RHODES

With a Note on the Immediate Death-Rate

By P. J. FISHER

National Collection of Type Cultures, Colindale Avenue, London, N.W. 9

SUMMARY: Cultures were dried in a vacuum over phosphorus pentoxide. At varying intervals up to 14 years tests were made on 2724 strains (representing over forty genera) and 83 % of cultures were found to be viable. *Staphylococcus*, *Sarcina* and *Micrococcus* were the genera most resistant to drying; *Vibrio* and *Neisseria* were among the least resistant.

Many methods have been described for the preservation of bacteria by drying (Hammer, 1911; Rogers, 1914, 1949; Swift, 1921, 1937; Flosdorf & Mudd, 1938; Rayner, 1943; Greaves, 1944; Stamp, 1947; Proom & Hemmons, 1949); these differ in the desiccating agent, menstruum for the bacteria, and in the complexity of the apparatus and manipulation needed. All the modern methods give satisfactory results; simplicity and efficiency are the only reasons for reporting the method to be described. The method is not original, but I cannot find any reference to it in the literature; Prof. A. Sordelli described it to Dr R. St John-Brooks and it has been in use in the National Collection of Type Cultures (N.C.T.C.) since 1934. Tubes were opened as cultures were required for distribution; there was no planned experiment, but over 15 years a mass of information has accumulated which is summarized in this paper.

METHODS

Drying. Cultures were usually grown on a solid medium for 24 hr.; cooked meat medium (Robertson's medium) was sometimes used for the anaerobes. Organisms were not dried until there was good growth; with actinomyces and mycobacteria this sometimes took a week or more.

Rimless tubes were specially made in two sizes (Poulton, Selfe and Lee Ltd.); the smaller (8 × 60 mm.) was a neat fit in the larger (10 × 150 mm.). The smaller tubes (Fig. 1 *a*) were plugged and sterilized, and until required for use were kept dry in an incubator. Identification data were written in indian ink on the small tube (white ink was unsatisfactory as it was affected by the P_2O_5). A small amount of P_2O_5 was put in the large tube; to do this without fouling the sides a glass rod and funnel with a stem 130 mm. long were used (Fig. 1 *b*).

A loopful of horse serum was deposited on the inner wall of the small sterilized tube and a loopful of growth was emulsified in it. The small tube was inserted into the wider tube containing P_2O_5 and the tight fit of the cotton-wool plug prevented the smaller tube from making contact with the P_2O_5 (Fig. 1 *c*). The outer tube was then constricted above the level of the wool plug

(Fig. 1*d*), and when cool was attached to a Hyvac pump. A good vacuum was obtained in 2–3 min. and the tube was sealed at the constriction (Fig. 1*e*); the vacuum was examined with a high frequency tester (W. Edwards and Co. (London) Ltd.). Cultures were stored in the dark at room temperature.

Recovery. The outer tube was opened and the inner tube removed; about 1 ml. of broth or other suitable liquid medium was pipetted into the small tube, and

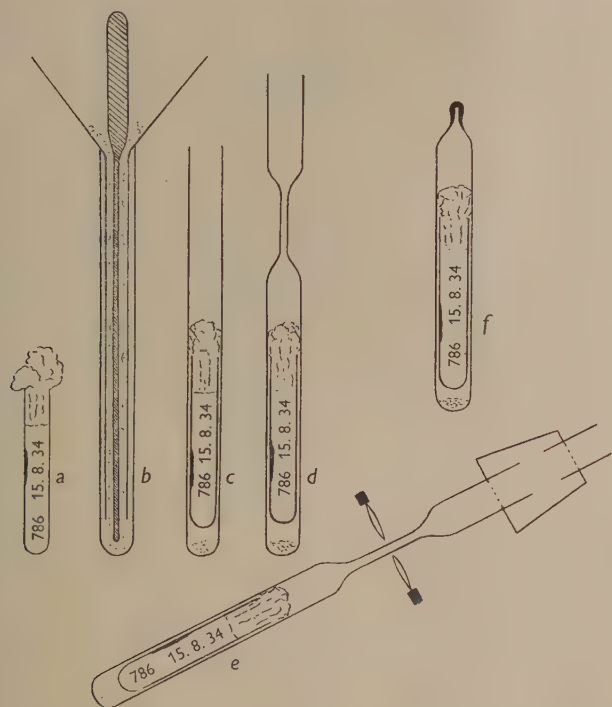


Fig. 1. Stages in the preparation of dried cultures. *a*, small sterile tube with identification data; *b*, funnel, and glass rod used for putting P_2O_5 at the bottom of the large tube; *c*, small tube in larger tube, the bottom of the small tube is not in contact with the P_2O_5; *d*, large tube constricted above the plug of the smaller tube; *e*, sealing off the outer tube while attached to the vacuum pump; *f*, the finished product.

incubated at the optimum temperature of the organism. When turbidity developed, usually within 24–48 hr., a subculture was made on solid medium. Incubation was continued for 10–14 days (21–28 days for actinomyces and mycobacteria) before discarding tubes that failed to show growth.

Material dried. Almost every bacterium in the N.C.T.C. was dried in duplicate between 1934 and 1939; afterwards two desiccates were made of each new strain (with the exception of *Leptospira* spp.) added to the Collection.

To keep up the stock, a new dried culture was made from the growth of a tube that had been opened. If the first tube failed to grow the second tube

was opened and, if that grew, two new desiccates were made. Generally, the first tube opened was picked at random; it might have been dried for less than a month or for many years. In 1948–9 some selection was made, and, to increase the number of cultures tested after longer intervals, the older tube was chosen.

Identification of cultures and growth. Until recently the depositor's determination was always accepted. Sometimes dried cultures were made from the original culture sent to the Collection. After recovery from the dried state the identity of the culture was confirmed by inspection of the growth and by stained smears. Because of lack of time and the necessary facilities, biochemical and fermentation tests were seldom employed.

RESULTS

Just under a quarter of the tests were made on cultures that had been dried for less than a year (Table 1). Up to 11 years the survival rates varied between 79 and 88 % of tubes opened, but after longer intervals there was a sharp decrease. A possible explanation of the poor results from cultures dried in 1934 is that serum was not used when the method was first employed.

Table 1. *Viability of bacteria at different intervals after drying*

Interval	No. tested	Contaminated	Viable	Percentage viable
Less than 1 month	92	3	80	87·0
1 month	1604	34	1413	88·1
1 yr.	1004	30	854	85·1
2 yr.	674	21	572	84·9
3 yr.	409	12	344	84·1
4 yr.	291	7	237	81·4
5 yr.	295	10	258	87·5
6 yr.	257	4	217	84·4
7 yr.	293	4	233	79·5
8 yr.	357	10	282	79·0
9 yr.	448	7	376	83·9
10 yr.	491	8	414	84·3
11 yr.	428	14	346	80·8
12 yr.	162	9	111	68·5
13 yr.	154	11	103	66·9
14 yr.	169	27	78	46·1
	7128	211	5918	

Gross contamination occurred in 211 (3 %) of tubes opened, and these are included among the non-viable; in a number (not recorded) of tubes lesser degrees of contamination made it possible to recover the organism originally dried. The greatest number of contaminated tubes was found in species of *Vibrio* (52 contaminated). In other groups contamination was associated with the necessity for enrichment of the media used for growth; *Haemophilus* (32), *Neisseria* (18), *Streptococcus* (18), *Mycobacterium* (12), and *Pseudomonas* (12).

The genera tested, and their survival after different intervals, are shown in Table 2 (for species see 'List of Species', 1948). In compiling this table an attempt

Table 2. *Survival of different genera after drying*

Genus	No. of strains tested	Period (years) between drying and test					Percentage of cultures viable
		< 1	1–4	5–9	10–14	< 1–14 (Total)	
		No. of cultures viable/no. tested					
Actinomyces (anaerobic)	29	9/11	19/37	7/9	3/4	38/61	62
Actinomyces (aerobic)	20	—	13/15	7/11	2/2	22/28	79
Nocardia	18	4/4	3/3	13/15	4/8	24/30	80
Streptomyces	34	13/13	20/21	16/19	2/2	51/55	93
Acetobacter	34	20/20	38/42	18/22	8/9	84/93	90
Pseudomonas	107	34/36	76/102	46/62	66/109	222/309	72
Vibrio	134	56/74	53/101	32/92	11/132	152/399	38
Xanthomonas	36	11/14	30/39	9/9	10/14	60/76	79
Mycoplana	1	—	—	0/2	—	0/2	—
Spirillum	4	1/1	1/1	0/1	1/1	3/4	—
Micrococcus	72	51/51	21/21	29/29	26/26	127/127	100
Sarcina	11	7/7	2/2	4/4	9/9	22/22	100
Staphylococcus	108	54/54	132/133	47/48	30/30	263/265	99
Neisseria	88	62/69	66/118	38/72	22/61	188/320	59
Lactobacillus	35	15/17	17/22	23/29	9/12	64/80	80
Leuconostoc	13	8/10	20/24	5/5	7/7	40/46	87
Streptococcus	316	398/445	567/610	171/178	129/136	1265/1369	92
Bacillus	240	55/69	87/95	169/183	122/128	433/475	91
Clostridium	24	6/11	12/17	8/13	1/3	27/44	61
Flavobacterium	7	7/7	12/14	3/3	0/1	22/25	88
Chromobacterium	26	7/10	22/27	9/16	8/14	46/67	69
Bacterium (coli-aerogenes)	113	69/69	75/78	70/71	55/59	269/277	97
Bacterium (*Erwinia*)	28	10/10	14/18	15/16	16/23	55/67	82
Bacterium (non-fermenting)	11	5/5	5/7	6/7	6/6	22/25	88
Bacterium (paracolon)	42	10/10	8/8	28/29	18/18	64/65	98
Bacterium (uncertain status)	67	37/46	31/44	32/39	23/30	123/159	77
Salmonella	246	29/29	70/73	144/146	69/76	312/324	96
Shigella	130	46/46	88/95	73/78	57/62	264/281	94
Proteus	104	52/54	48/48	39/44	72/77	211/223	95
Mycobacterium	106	52/55	49/61	50/71	51/61	202/248	81
Corynebacterium	154	71/72	98/104	97/102	52/59	318/337	94
Erysipelothrix	27	2/2	5/6	13/13	17/17	37/38	97
Zopfius	7	1/2	1/1	7/7	6/6	15/16	94
Actinobacillus	10	11/13	18/24	3/5	7/11	39/53	74
Brucella	58	40/48	33/46	26/31	55/72	154/197	78
Haemophilus	110	191/237	159/199	33/56	15/26	398/518	77
Pasteurella	58	31/34	59/62	35/42	33/37	158/175	90
Pfeifferella	10	—	7/8	5/6	5/7	17/21	81
Fusiformis	17	3/5	9/28	2/7	1/9	15/49	31
Azotobacter	15	2/5	—	5/10	6/13	13/28	46
Leptothrix	3	—	—	0/5	—	0/5	—
Microspira	1	0/1	0/1	1/3	—	1/5	—
Photobacterium	9	4/16	1/3	3/5	0/2	8/26	31
Propionibacterium	11	5/5	5/5	16/17	2/2	28/29	97
Pleuro-pneumonia	6	2/6	10/12	5/6	5/6	22/30	73
Vibriothrix	2	—	—	2/2	—	2/2	—
Rhizobium	20	1/1	2/2	2/9	9/15	14/27	52
Sporocytophaga	1	1/1	—	—	1/1	2/2	—
Thiobacillus	1	0/1	1/1	0/1	1/1	2/4	—

has been made to place species in the appropriate genus; for example, strains received as *Salmonella morganii* are shown under *Proteus*, but because complete characterization was not undertaken in the Collection, some strains may have been seriously misplaced. With most genera a high proportion of tubes were viable up to 10–12 years and little comment is needed. *Pseudomonas trifoliorum*, *Vibrio cholerae*, *Brucella tularense* and *Mycobacterium murium* were species that did not survive for long periods. Four strains of *Chromobacterium violaceum* soon died out but another strain lived for 5 years.

In general, the *Bacillus* group dried well but *B. repens* was an exception; of two strains tested, one (N.C.T.C. 7126) was not recovered from either tube, and the other (N.C.T.C. 4820) only from two of four tubes. Most *Clostridium* species survive well in meat cultures, consequently few were dried. Four strains of *Cl. acetonigenum* were dried but only three of eleven tubes were alive; sand cultures (Thaysen, 1924) of this organism, received from Dr A. C. Thaysen, grew after storage for at least 10 years.

The results obtained with *Rhizobium* spp. must be interpreted with caution; when these strains were received bodies resembling spores were seen in stained films and doubt was felt about the correctness of the determination. Unfortunately, before drying the cultures films were not stained specifically for spores. When tested, most tubes gave a growth of *Bacillus circulans*, some a mixture of *Rhizobium* and *Bacillus circulans*.

Fungi and yeasts were dried by the same method and the results have been reported (Rhodes, 1950).

Effect of storage temperature

An experiment was made to see if dried cultures could be sent by post to the tropics. Six tubes of *Staphylococcus aureus* and six tubes of *Vibrio cholerae* were dried and kept at 37°. Two years later all tubes of the *staphylococcus* were viable; all those of the *vibrio* were dead. These results are not included in Tables 1 or 2.

DISCUSSION

Survival rates in the tables refer to the number of tubes that gave growth on test, and not to the proportion of organisms that were viable. Experiments made recently by P. J. Fisher show that the method (slightly modified in that a measured volume was dried) may kill from 20 to 99 % of bacteria in suspension during the drying period. With such a high immediate death-rate it is essential that the suspension to be dried should be heavy and consist mainly of living organisms. It follows, therefore, that the optimal time to dry the culture is at the end of the logarithmic phase. Disregard for this principle, as in the chromobacteria where growth was continued until pigment had developed, and the use of the depositor's original cultures, may account for the poor results obtained with species such as *Chromobacterium violaceum* and *Mycobacterium murium*.

In general, the results agree with those of Stamp (1947) and of Proom & Hemmons (1949) who found *Vibrio cholerae* and the pathogenic species of

Neisseria viable only for short periods, while streptococci and staphylococci survived the longest time tested.

It is to the interest and encouragement of Dr R. St John-Brooks, who started drying cultures by the method outlined in this paper, that I owe my thanks.

A Note on the Immediate Death-Rate

By P. J. FISHER

Without modification, Sordelli's method cannot be controlled by viable counts of the organism before and after drying. To do this the volume of suspension must be increased, and this will inevitably decrease the rate of drying. Experience has shown that an increase in the drying time is accompanied by an increase in the killing effect of the process. It follows, therefore, that the death-rates reported in this note are probably greater than those obtained in the routine use of Sordelli's method as described by Miss Rhodes.

Table 3. *Viable counts before and after drying*

Organism	Strain N.C.T.C. no.	Viable count/ml. Before	Viable count/ml. After	Death-rate (%)
Bacterium coli	86	38×10^5	24×10^5	37
Streptococcus pneumoniae	2428	134×10^5	110×10^5	18
Vibrio cholerae	7270	80×10^5	83×10^3	99
Neisseria gonorrhoeae	6822	40×10^4	32×10^3	92

A measured volume of suspension was dried in vacuum over P_2O_5; using the roll-tube method (Wilson, 1922) viable counts were made of the suspension to be dried and of the dried product after reconstitution to the original volume. Table 3 shows the results of four typical experiments, two made with species known to survive for a long time, and two with species that do not dry well. The immediate death-rate, calculated from the counts made before and 24 hr. after drying, varied from 18 % with *Streptococcus pneumoniae* to 99 % with a freshly isolated strain of *Vibrio cholerae*.

REFERENCES

Flosdorf, E. W. & Mudd, S. (1938). An improved procedure and apparatus for preservation of sera, microörganisms and other substances—the Cryochem-process. *J. Immunol.* **34**, 469.

Greaves, R. I. N. (1944). Centrifugal vacuum freezing; its application to the drying of biological materials from the frozen state. *Nature, Lond.*, **153**, 485.

Hammer, B. W. (1911). A note on the vacuum desiccation of bacteria. *J. med. Res.* **24**, 527.

List of species maintained in the National Collection of Type Cultures (1948). *Mem. med. Res. Coun., Lond.*, No. 21.

Proom, H. & Hemmons, L. M. (1949). The drying and preservation of bacterial cultures. *J. gen. Microbiol.* **3**, 7.

Rayner, A. G. (1943). A simple method for the preservation of cultures and sera by drying. *J. Path. Bact.* **55**, 373.

Rhodes, M. (1950). Preservation of yeasts and fungi by desiccation. *Trans. Brit. mycol. Soc.* **33**, 35.

Rogers, L. A. (1914). The preparation of dried cultures. *J. infect. Dis.* **14**, 100.

Rogers, L. A. (1949). An apparatus for the desiccation of cultures. *J. Bact.* **57**, 137.

Stamp, Lord (1947). The preservation of bacteria by drying. *J. gen. Microbiol.* **1**, 251.

Swift, H. F. (1921). Preservation of stock cultures of bacteria by freezing and drying. *J. exp. Med.* **33**, 69.

Swift, H. F. (1937). A simple method for preserving bacterial cultures by freezing and drying. *J. Bact.* **33**, 411.

Thaysen, A. C. (1924). A description of two methods for the preservation of stock cultures of micro-organisms. *J. Inst. Brew.* **30** (N.S. **21**), 349.

Wilson, G. S. (1922). The proportion of viable bacteria in young cultures with especial reference to the technique employed in counting. *J. Bact.* **7**, 405.

(*Received* 19 *January* 1950)

Further Observations on the Motility of *Proteus vulgaris* Grown on Penicillin Agar

By A. FLEMING

The Wright-Fleming Institute of Microbiology, St Mary's Hospital, Medical School, London, W. 2

SUMMARY: The movement of the enormously enlarged filaments of *Proteus vulgaris* grown on penicillin agar ceased, or was slowed down, by screening the culture from the radiant heat of a microscope lamp. Organisms stimulated by heat after resting a short time in this way moved more rapidly than before. The increased activity was maintained for longer periods with longer rests, the increment in the period of activity increasing gradually as the resting period increased. In the condition of the test, rests of 60–120 sec. produced a maximal response.

Repeated heat stimulation rapidly exhausts the capacity of the organisms to maintain a steady rate of movement. Under constant stimulation organisms maintain a fairly constant rate for some hours and then slow considerably as though they or their flagella were exhausted. A small decrease in radiant heat energy can induce a reversal in the direction of movement.

Active flagella attached to immobile organisms react to heat stimuli in the same way.

The effect of penicillin on the morphology of *Proteus vulgaris* has often been described. With concentrations between 3 and 10 units (u.)/ml. the filamentous forms of the microbe become very long and many of them become very distorted.

In a previous article I and my co-workers (Fleming, Voureka, Kramer & Hughes, 1950) made observations on the motility of *Pr. vulgaris* grown on penicillin agar and examined by phase-contrast microscopy. We showed that when radiant heat from the microscope lamp was cut out by the insertion of a heat filter, the motility of the greatly enlarged filaments of *Pr. vulgaris* ceased or slowed down, and that the time during which an organism could maintain its maximum activity depended on the period of rest which preceded such movement. These findings applied also to flagella belonging to filaments which were themselves motionless. The present paper records an extension of these observations.

Technique. The organisms were grown in slide cultures as previously described. Cultures on agar containing 5 u./ml. of penicillin were incubated at room temperature. I was especially interested in the long filaments which were coiled like a watch-spring and whose only movement was a simple rotation. These could be watched for hours in the same field of the microscope. A sure way of obtaining these forms was to plant *Pr. vulgaris* in a thick streak across the cover-slip, and after this had dried to put two or three drops of penicillin agar at 50° on the cover-slip and immediately invert it on a slide. Along the edges of the streak there were always many 'watch-springs' to be found.

The heat filter was that supplied by Kodak for their 'Retina' lantern; it absorbed only about 10 % of the light but about 80 % of the radiant heat.

The flagellar movements or the rotations of the coiled filaments were timed by an assistant who noted to the nearest second the intervals called by the observer at the microscope. A better way was to use a wire recorder; the observer at the microscope talks into the microphone and then the record is played back and timed. This has the advantage that the times could be checked if there was any doubt.

Movement of the watch-spring forms. The rate at which the coils rotate varied enormously with different cultures and with different organisms in the same culture. The rotations may be rapid—less than 1 sec.—or they may be very slow, up to 30 sec. or more.

In the older slide cultures almost all the organisms are immobile and only the 'watch-springs' continue their rotary movements. It seems strange that this should be so except that possibly in their rotary movement these organisms have worn a groove in the agar so that they are actually moving in a thin layer of fluid. Thus, they would have little resistance to overcome and so would be able to continue movement for a longer period than the others which, in their enlargement, became caught between the agar and the cover-slip.

The effect of a heat filter. The effect of decreasing the radiant heat varies somewhat with different cultures and different organisms. Occasionally it has little effect on the rate of rotation of the 'watch-springs', but usually the organism either stops or slows down very considerably. The cessation of movement may be only temporary, and after a period varying from a few seconds to some minutes the rotation recommences slowly and eventually reaches about the same rate as it would had there been no heat filter for a considerable time. The effect on the visible flagella is the same. They usually stop completely and then start moving slowly.

The flagella are extraordinarily sensitive to the variation of the radiations caused by the interposition of the heat filter. The mere passage of the filter across the path of light so that it is effective for not more than one-fifth of a second is often sufficient to stop or slow down the movement of the flagella. The introduction of a heat filter may also induce a reversal of the directional movement of many filaments.

Effect of removing the heat filter. After a short latent period there is a violent movement of the flagella and the rate of rotation is markedly increased. The rotation is maintained at the increased rate for a variable time and then decreases (Table 1). Rotation usually slows to below its regular speed, as if the organism were exhausted by the violent effort and then gradually picks up again to its normal speed, which may be maintained for a long time.

Effect of inserting the heat filter for varying times. Fleming *et al.* (1950) showed that the number of revolutions which the watch-spring forms make at increased speed depends on the time during which the radiant heat is excluded. A very much larger series of observations has amply confirmed this relationship. Table 1 shows the results obtained with a single organism, stimulated to increased movement by exposure to radiant heat after varying periods without it.

When the time of rest is plotted against the number of revolutions before

slowing, a regular curve is obtained (Fig. 1). There is a rapid rise in the number of revolutions following increases in the period of rest up to 20 or 30 sec. and then the curve flattens out and there is little difference between a rest period of 1 and 2 min.

Table 1. *Rate of movement of a 'watch-spring' filament of* Proteus vulgaris *after varying periods of rest*

Period of screening from radiant heat (sec.)	Period of successive revolutions upon application of radiant heat (sec.)
180	3 3 3 3 2 3 3 2 2 3 3 2 2 3 3 2 4 4 3 3 4 5 stopped
2	4 3 2 2 2 4 4 13 stopped
75	3 2 2 2 2 3 2 3 2 3 2 2 3 3 3 2 2 4 4 3 3 3 9 stopped
10	4 2 2 3 2 2 3 2 2 2 2 3 5 8 stopped
5	4 2 2 3 2 2 3 4 8 25 stopped
1	5 3 12 16 stopped
75	3 2 2 2 3 2 2 3 2 3 2 3 2 3 4 3 4 4 4 4 4 3 5 11 stopped

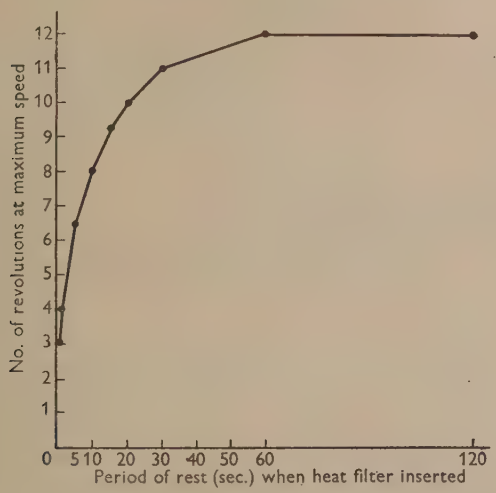


Fig. 1

The effect of repeated heat stimulation. In the experiment recorded in Table 1 there was apparently no exhaustion after repeated stimulation. It is usual, however, especially in older cultures, to see, after intermittent heat stimulation, that the organism becomes gradually more sluggish. In one experiment (Table 2) the time taken for each revolution increased, with the heat filter in, from 2·8 sec. to 14 sec., and with the heat filter out from 1·6 to 9 sec.

Period during which an organism can maintain its activity. A rotating watch-spring form in a slide culture which had grown overnight at room temperature was timed at intervals for 5 hr., during which time it was exposed to radiant heat from the microscope lamp. The period of each of ten consecutive revolu-

Table 2. *Exhaustion of an organism after repeated heat stimulation to increased activity*

Position of heat filter	Period of successive revolutions (sec.)	Average time in seconds for each revolution: Filter in	Filter out
In, at beginning of observation	3 3 2 3 3 3 3 3 2 3	2·8	—
Out after 5 min.	40 (25 revolutions)	—	1·6
In after 2 min.	6 6 5 5 4 5 5 4 5 4	4·9	—
Out after 4 min.	4 4 4 5 5 4 5 5 6	—	4·7
In after 47 sec.	10 10 10 9 9	9·8	—
Out after 48 sec.	5 4 6 5 5	—	5·0
In after 25 sec.	14 11 11	12·0	—
Out after 36 sec.	7 7 8 8 8	—	7·4
In after 38 sec.	15 14 13	14·0	—
Out after 42 sec.	10 8	—	9·0
In after 18 sec.	∞ (organism motionless)	—	—

Table 3. *Activity of an organism exposed constantly to the radiant energy of the microscope lamp*

Period of exposure to radiant heat (hr. min.)		Period of ten consecutive revolutions (sec.)	Average period of revolution (sec.)
0	5	6 7 6 5 6 7 6 6 6 6	6·1
0	40	7 6 6 5 6 6 5 6 6 6	5·9
1	10	6 6 5 4 6 5 5 7 5 5	5·4
2	10	6 6 8 6 6 6 7 6 7 7	6·5
3	0	7 7 8 6 7 7 7 7 8 7	7·1
3	25	8 8 8 9 7 7 7 8 8 8	7·8
4	0	11 12 10 12 10 11 13 12 12 11	11·4
5	0	15 16 15 18 21 16 15 19 16 16	16·7

tions was observed at intervals, the first observation being 5 min. after exposure to the light, when the rotation rate had become regular (Table 3).

Then the heat filter was inserted to ascertain whether, after a rest, the organism would regain its maximum speed. As soon as the filter was inserted movement ceased, and after about 10 sec. it was resumed but very slowly. After the filter had remained in for 40 min. the rate was an average of 25·6 sec./rev. The filter was removed after 45 min. and the average time for the first 5 rev. was 13·8 sec., which was faster than that observed nearly an hour before; but the second 5 rev. averaged 27·2 sec. which was even slower than the rate when the heat filter was in. Ten minutes later, exposed to the lamp without a filter, each revolution took 28·5 sec. These observations show the gradual development of fatigue in the organism after about 3 hr.

Constancy of the rate of movement. The figures given in Table 3 show that there is a very considerable constancy in the rate of revolution at any one time. At one period the organism was timed for fifty consecutive revolutions. The times were, as usual, recorded to the nearest second.

The results were as follows: 2 rev. were made in 6 sec. each; 20 rev. were made in 7 sec. each; 21 rev. were made in 8 sec. each; 7 rev. were made in

9 sec. each. It is clear, therefore, that the organism maintains a fairly constant rate of rotation.

Reversal of direction of movement. The insertion of a heat filter not only can immediately stop flagellar movement in slide cultures but can also induce a reversal of the direction of movement. This reversal was not seen in coiled forms and only very seldom in the very common forms, which were bent double and were travelling along with the bend foremost. It was especially noticeable in the short- or medium-length filaments which had remained more or less straight. A filament would be travelling in a certain direction across the field, say to the right. When the filter was inserted it stopped and after a second or two moved slightly to the left. If the filter remained in it would after a short delay move slowly to the left. When the filter was removed as soon as the first movement to the left was seen, the organism would dart forward in that direction. If then the filter was reinserted, the organism would stop and again reverse its direction. This to-and-fro control of the organism by manipulating the filter could be kept up for a long time. When the organism was escaping from the microscope field it could be stopped and brought back by the insertion of the filter.

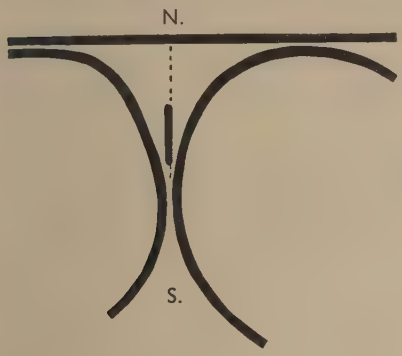


Fig. 2

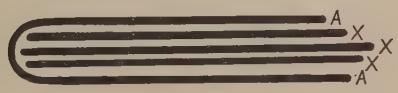


Fig. 3

Fig. 2. Reversal of direction. Dotted line shows the path of the organism.

Fig. 3. Reversal of direction. Under appropriate heat stimulus the bent filament *AA* maintains its direction of movement, while the straight filaments *XXX* reverse their direction of movement and leave the loop.

I observed one very good example for a considerable period. A short organism only about twice as long as a normal *Pr. vulgaris* was moving backwards and forwards in a north-south direction in a space only about twice as long as itself. It was enclosed between filamentous forms as shown in Fig. 2. With the heat filter in it moved gently up and down. The flagella could be seen waving gently on both sides behind the organism. The filter was removed when it had just started to move north. Almost immediately the flagella became violently active and the organism was driven against the north barrier where it remained motionless while the flagella continued in active motion.

The filter was again inserted, the flagellar motion ceased and then it slowly returned south. Then it continued to move gently north and south while the filter was in. When it reversed its motion the flagella which had been active at one end remained stationary, but flagellar activity could be seen at the other end and the bacillus was pushed between the groups of resting flagella which appeared to fold themselves back along the body. This was repeated many times, always with the same result.

It is very common in these slide cultures to see loops of organisms moving together. In Fig. 3 there is one on the outside bent over in the middle (*A*) and inside this there are three straight rods (*XXX*). This loop could be emptied of its straight elements by inserting the heat filter which induced reversal of direction in the straight rods while the bent rod (*A*) continued in its original path.

The insertion of the heat filter stops flagellar movement, and it is evident that it is then that there is some impulse for the organism to reverse its direction.

Movement of flagella attached to organisms which remain stationary

So far we have been dealing with the actual movement of organisms. At the same time the flagella of these organisms could be clearly seen and, as we have stated in a previous communication (Fleming *et al.* 1950), flagellar movement may precede movement of the organism. In the slide cultures it is

Table 4. *Duration of movement of flagella attached to twenty different immobile organisms after varying periods of rest from heat stimulation*

Organism no.	Period that culture was screened from radiant heat before observation (sec.)						
	1	5	10	15	20	30	60
	Duration of flagellar movement (sec.)						
1	7	20	—	25	—	37	43
2	6	14	—	—	—	31	—
3	—	10	18	—	19	—	30
4	—	13	16	18	20	—	18
5	7	16	22	—	20	—	29
6	—	17	23	—	—	—	—
7	—	16	18	—	23	—	27
8	—	14	21	—	25	—	23
9	—	—	31	—	—	36	47
10	15	—	—	37	—	—	—
11	—	27	46	—	—	—	61
12	—	14	22	34	—	46	60
13	—	15	18	20	21	26	29
14	—	15	17	—	27	30	37
15	—	21	25	35	31	37	—
16	—	22	29	—	33	36	43
17	10	24	—	38	—	47	52
18	15	23	—	—	—	47	54
19	20	29	34	37	39	45	50
20	12	19	23	23	25	28	—

common to find active flagellar movement while the organism is stationary. Just like the movement of the whole organism, this flagellar movement continues for a longer time after a longer period of rest; and, further, the latent period, i.e. the time between the removal of the heat filter and the commencement of active flagellar movement, is longer with the shorter periods of rest. Many observations have confirmed these findings. Table 4 gives the actual periods of flagellar movement after various periods of rest or comparative rest when the heat filter was inserted.

Naturally with different individual organisms the times varied, but there is a very good agreement in the relative periods of movement after long and short periods of rest.

Latent period between the time the heat filter was removed and the commencement of violent flagellar movement. Extended observations have confirmed our statement that with short periods of rest the latent period was longer. I cannot go further than this. With more elaborate timing apparatus it would be possible to give accurate figures but there is no doubt that with short periods of rest, e.g. 1 or 2 sec., the latent period is much longer than with rest periods of 30–60 sec.

DISCUSSION

All the observations were made on *Proteus vulgaris* grown on penicillin agar; these are not normal organisms. The penicillin allows growth but inhibits cell division so all the filaments on which observations were made were compound organisms. They possessed very long flagella which wound themselves into undulating 'ropes', easily visible by phase-contrast microscopy.

There are in these cultures long filaments which move with an undulating motion exactly like a snake, but there are many others in which no motion of the body can be seen but which have active flagella which could be responsible for the movement. The flagella respond to the stimulus of very small changes in radiant heat exactly as do the motile organisms. It may be that the flagella are a type of extra-corporeal muscle which will respond to stimuli just as do muscle fibres. Heat is the only stimulus which has been investigated, but it will be interesting to try various forms of electric stimulation.

The amount of energy used in flagellar movement must be enormous, but the organisms can maintain their movements for several hours with little diminution in the rate of movement, although later the rate rapidly diminishes as if the organism was becoming exhausted.

I wish to thank Mr John L. Smith for the gift of a wire recorder which made this work easier.

REFERENCES

Fleming, A., Voureka, A., Kramer, I. R. H. & Hughes, W. H. (1950). *J. gen. Microbiol.* **4**, 257.

(*Received* 17 *February* 1950)

Some Factors Affecting the Activation of Virus Preparations Made from Tobacco Leaves Infected with a Tobacco Necrosis Virus

By F. C. BAWDEN and N. W. PIRIE

Rothamsted Experimental Station, Harpenden, Hertfordshire

SUMMARY: Preparations of the Rothamsted tobacco necrosis virus were made by the ultracentrifugation of sap from infected tobacco leaves after a preliminary concentration by freezing. Not all the anomalous nucleoprotein in these preparations was infective, and the products were fractionated by differential ultracentrifugation at lower speeds and by precipitation at pH 4 in the presence of sedimentable protein from uninfected leaves. The more readily sedimentable and precipitable material carried with it most infectivity, whereas the other material had the greater serological activity.

Preparations made quickly from freshly expressed sap were less infective than those made from sap that had been frozen or allowed to age for a few days. The extent of the activation produced by these treatments depended on the physiological condition of the infected leaves.

As much virus could be extracted from the leaf residues as occurred in the sap. The infectivity of this residual virus depended on the medium used for its extraction.

It is suggested that much of the infectivity of this virus in sap is acquired during or after extraction from the leaf, but the relationship between the particles with different sizes and properties remains uncertain.

Smith & Bald (1935) identified tobacco necrosis as a virus disease and, since then, several causative viruses have been differentiated by their serological reactions (Bawden, 1941) and by their different properties *in vitro* (Bawden & Pirie, 1942, 1945*a*). A crystalline nucleoprotein has been isolated from plants infected separately with each virus, and with all except one there is much evidence identifying the protein with the virus, although the precise relationship between the two remains unestablished. The virus we have called the Rothamsted culture, however, is exceptional, and the status of the crystalline nucleoprotein obtainable from plants infected with it is very uncertain. All highly infective preparations were demonstrably inhomogeneous, and there was little positive evidence about the relationships between the various components. Increasing the homogeneity of the preparations of nucleoprotein decreased the infectivity, but the significance of this was uncertain as the virus readily loses infectivity during treatments that appear not to affect other tobacco necrosis viruses.

When preparations of the Rothamsted tobacco necrosis virus made in 1945 were ultracentrifuged they gave pellets that were crystalline; when these pellets were washed quickly with successive small lots of water, the material extracted in the successive washes was, weight for weight, progressively less infective. The most slowly dissolving material was the most highly crystalline and usually had the highest precipitin titre, but was the least infective. This phenomenon has three equally plausible interpretations: infective and non-

infective virus particles may resemble one another and crystallize together, but dissolve at different rates; infective particles may not crystallize, and the crystalline material may all be non-infective, though derived by some modification of virus particles; the crystalline material may all be non-infective and, although specific to infected plants, may be distinct from the virus particles and not derived from them. Our recent experiments have not decided between these various interpretations, but they have given further information on the conditions that lead to variations in infectivity, with the result that much more infective preparations can now be made. As other viruses may behave similarly, it seems worth recording some of the anomalous phenomena we have encountered with the Rothamsted tobacco necrosis virus, although few are definitely interpretable and many are not consistently reproducible.

MATERIALS AND METHODS

The virus used was derived by repeated subculture from the stock we used previously under the name Rothamsted. Transfers were usually made with a bulk inoculum, but at irregular intervals fresh stocks were built up from virus derived from single local lesions. There is no proof that we have worked continually with the same virus or virus strain, but there is no evidence to the contrary; nor is there any evidence that our stocks contained more than one tobacco necrosis virus, although this also cannot be disproved. The most that can be said is that all the inocula produced material that was serologically related and which shared the same general properties and crystalline characters. The virus was propagated in tobacco plants, *Nicotiana tabacum* var. White Burley, which were dusted with celite before the upper surfaces of their leaves were rubbed with infective sap. We (Bawden & Pirie, 1945*a*) have previously described large seasonal differences in the susceptibility of plants and stated that during summer yields are low and purification difficult. These seasonal differences are correlated with variations in light intensity (Bawden & Roberts, 1947, 1948). Susceptibility in summer is much increased when the plants are raised under shade and placed in the dark for 2–3 days immediately before they are inoculated. By doing this, high yields of virus have been obtained throughout the year and purification has been greatly facilitated.

Infectivity tests were made by the half-leaf method using French bean, *Phaseolus vulgaris* var. Prince, half-leaves being rubbed as evenly as possible with the forefinger wet with the inoculum. Preparations to be compared were tested at two dilutions, usually differing by a factor of ten, and each dilution was inoculated to at least six half-leaves. The inocula were systematically varied over the half-leaves so that none appeared twice on the same plant, all possible combinations occurred on the same plant, and each was applied to an equal number of left- and right-hand halves.

Precipitin titres were determined by adding 1 ml. of virus preparation at various dilutions to each of a series of tubes containing 1 ml. of antiserum at a constant dilution, usually 1/50. The tubes were immediately placed in a water-bath held at 50°, with the fluid columns half-immersed so that convection currents kept the mixtures continuously moving. The precipitin titre was taken

as the smallest amount of antigen that produced a precipitate visible to the eye after 3 hr. in the water-bath. Antisera were prepared in rabbits by the intravenous injection of virus preparations in various stages of purification and possessing various degrees of infectivity. Fully-crystalline preparations with little infectivity were most active antigenically, equal weights of these producing antisera with higher precipitin titres than those obtained by injecting more infective but less homogeneous preparations, but no qualitative differences have been noted between antisera produced against the various types of antigen.

Preliminary treatments

The Rothamsted tobacco necrosis virus loses infectivity more rapidly in sap than when purified, and it seemed possible that the heterogeneity of purified preparations might be, at least in part, an artefact caused by the exposure of virus particles to the conditions obtaining in sap. Some sap components of small molecular weights cause particles of tobacco mosaic virus to aggregate, and *in vitro* changes with this virus can be diminished by removing such components before the infected leaves are macerated (Bawden & Pirie, 1945*b*). In preparing unaggregated tobacco mosaic virus, convenient treatments are to use the leaf cells as dialysis membranes after destroying their osmotic control by freezing or exposure to chloroform. In preparing this tobacco necrosis virus, however, such treatments are inappropriate as they themselves cause much loss of infectivity. In other attempts to lessen deleterious changes, sap was cooled, the various treatments were made with as little delay as possible and the preparations kept as cold as possible throughout. So far from increasing infectivity, this treatment gave a less infective product than that made from sap kept at room temperature and fractionated over a longer period. It is obvious that particles of the virus are affected in various ways in sap, but the changes that predominate at first increase rather than decrease infectivity. Our most infective preparations were made by using ultracentrifugation to sediment the virus and delaying its application for some time after the sap had been expressed from macerated leaves.

Small quantities of the virus are readily prepared by the direct ultracentrifugation of clarified sap, but the virus content of sap is small and this method is laborious when quantities sufficiently large for chemical study are needed. In our previous work, the virus in sap was first concentrated by a preliminary precipitation with ammonium sulphate, but this is undesirable as the required concentration of salt causes considerable inactivation. Attempts at ultra-filtration on cellophan by the method described by Paterson, Pirie & Stableforth (1947) were unsuccessful, because the sticky precipitate that separates from sap clogs the filtering surface. Although the removal of other sap components would be advantageous, it is not essential at this stage. The primary purpose of the treatment is simply to decrease the volume of fluid so that more virus can be ultracentrifuged. The method we found most suitable was to remove most of the water by freezing.

The removal of water by freezing is used in several technical processes (apple-jack is one well-known product) and it has occasionally been used to

concentrate proteins (Mellanby, 1908; Palmer, 1934; Bawden & Pirie, 1937), but it has been less used than it merits as a routine method for concentrating unstable substances. The process has been long known and probably originated from accidental observations by arctic travellers; thus, Gerart de Veer (1596), who wintered with Barents on Nova Żembla, remarks 'wee were forced to melt the Beere, for there was scant any unfrozen Beere in the Barrell, but in that thicke Yeaste that was unfrozen lay the strength of the Beere, so that it was too strong to drinke alone, and that which was frozen tasted like water'. Glauber (1658) recognized that only the water went into ice when dilute acetic acid was frozen and that stronger acid could be made by separating the unfrozen part. Boyle (1683) concentrated leaf extracts, in addition to beer and sea water, and concluded that the flavoured and coloured constituents were concentrated in the unfrozen part. His experiments refuted the statements of Aristotle and some medieval writers that the unfrozen part had the physical form of the plant from which the extract came; on all these issues our results confirm those of Boyle.

Preparation of infective virus in bulk

Leaves are picked 6–10 days after inoculation when they are well covered with lesions, and the main veins are cut away to avoid dilution with juice of a low virus content. The laminae are minced in a domestic meat mincer, and the sap expressed by squeezing through madapollam; the fibrous residue is minced and pressed again. After clearing by centrifuging for 10 min. at 7000 r.p.m., the sap is poured into a tray and placed in the freezing chamber of a refrigerator. Freezing proceeds from the surfaces, and the fluid collects in the centre and in spaces between ice crystals. These spaces remain connected until about 90 % of the water is frozen. Freezing is then discontinued, for otherwise the separation of the frozen and unfrozen parts becomes more difficult. The ice-block is broken by pounding through a grid with 1 cm. holes mounted in a metal funnel, and centrifuged immediately. With quantities of 300 g. or more, a basket centrifuge is convenient, but the separation can be done readily with a bucket-type centrifuge, by fitting perforated containers into the buckets. We have used household plastic cups without handles and with ten to twenty 1 mm. holes drilled in and around the bottom of each cup. The cups fit into the metal buckets or glass centrifuge tubes but are prevented by their conical shape or a flared rim from slipping right in. A few minutes' centrifugation at 3000 r.p.m. separates the fluid from the ice, and about one-sixth of the original volume of sap is obtained at the first run. This will usually contain about nine-tenths of the total virus, but a second run is advisable to ensure that this is so. If more than 90 % of the water has been allowed to freeze, much of the fluid will be contained in isolated pockets within the ice and will not be liberated until there has been extensive thawing during centrifugation. This condition can be recognized by the presence of coloured droplets within the ice at the end of centrifugation. With infective sap, virus and colour concentrate together, and the diminution in the colour of successive thawings is a reliable guide to virus content. If a second extract contains

appreciable quantities of virus, it can be concentrated by repeating the freezing.

All the constituents of the sap are concentrated by the freezing, and the density and viscosity are so increased that sedimentation in the ultracentrifuge is delayed. The fluid is therefore dialysed for a few hours against distilled water, when much of the low molecular-weight protein precipitates and can be removed by low-speed centrifugation. The clarified supernatant fluid is then centrifuged for 30–40 min. at 40,000 r.p.m. (80,000 *g*) in a centrifuge of the type described by Masket (1941), which sediments all the virus into compact pellets. In summer the pellets at this stage are usually brown and opaque, but in winter they may be yellow and almost transparent, with a crystalline fringe. The pellets are collected into one tube, mixed intimately with a volume of water equal to about one-hundredth that of the original sap, and centrifuged at 7000 r.p.m. (6000 *g*) after standing for a few hours. Some virus usually remains in the insoluble residue, which is therefore extracted a second time with water. The pooled extracts are again ultracentrifuged, when the pellets are clear, pale brown or yellow, with a definite crystalline fringe. The pellets are suspended in water and left for a few hours before insoluble material is removed by centrifuging at 7000 r.p.m.

The preparations at this stage are only faintly opalescent at concentrations around 5 g./l.; if they are not colourless, sedimentation for a third time will make them so and increase both their infectivity and serological activity. Additional centrifugations at 40,000 r.p.m., however, produce no useful fractionation, as all the material present sediments and redissolves in water. Nevertheless, the preparations are far from homogeneous, and they can be separated into fractions containing materials that have different ratios of infectivity to serological activity. Some separation is produced by centrifuging at 20,000 r.p.m. (22,000 *g*), even though our centrifuge is not well adapted to such separations because the tubes are inclined at only 10° to the axis. The results of one experiment, in which 7·5 ml. of a 1 g./l. virus solution was kept at 20,000 r.p.m. for 30 min. and then slowed to a stop in 15 min., will illustrate the kind of separation achieved. The contents of the tube were separated into three parts: the top 6·5 ml. of fluid, which was siphoned off immediately the centrifuged stopped; the bottom 1 ml. of fluid, near to or in contact with the pellet, which was decanted; and the pellet, which was dissolved in water. The three fractions contained 3, 1·7 and 2·8 mg. of solid matter respectively, and did not differ appreciably in serological activity, all three precipitating with antiserum to a dilution of 9 mg./l. Their relative infectivities, however, differed considerably; the mean numbers of lesions produced per half bean leaf by the materials in the three fractions were respectively 54, 79 and 110 at 2 mg./l., and 14, 30 and 41 at 0·2 mg./l.

Similar results were obtained in many other experiments, although sometimes the material that compacted into a pellet from preparations centrifuged for 30 min. at 20,000 r.p.m. was less active serologically than that in the supernatant fluid, despite its greater infectivity. When the material in the pellets was dissolved and centrifuged at 20,000 r.p.m. it again partitioned, but there

was little fractionation; the two parts had similar infectivities and serological activities. However, by repeating the centrifugation on the more slowly sedimenting material, this could be separated into fractions with differing infectivities. When the uncompacted material from several tubes centrifuged at 20,000 r.p.m. was pooled, concentrated by sedimentation at 40,000 r.p.m., and then again centrifuged at 20,000 r.p.m., the material remaining in the upper fluid was sometimes less than a tenth as infective as that in the pellet compacted in the first centrifugation at 20,000 r.p.m., although less was needed to give a visible precipitate with antiserum. When the material that has failed to compact twice at 20,000 r.p.m. is sedimented at 40,000 r.p.m. it gives pellets that are crystalline throughout, whereas pellets from the more infective material that compacts at 20,000 r.p.m. are largely amorphous with a crystalline halo. Pellets of both kinds of material can be fractionated by washing rapidly, with successive small lots of water, in the manner previously described (Bawden & Pirie, 1945*a*) when the most rapidly dissolving part from each type of pellet is, weight for weight, more infective than the more slowly dissolving parts.

After the most infective fractions have been separated by centrifugation at 20,000 r.p.m., they are still not homogeneous. This is shown by acidifying to pH 3·5–4·5, when part of the material precipitates and serological activity and infectivity partition unequally between the precipitated and soluble parts. If the suspension is quickly neutralized, the precipitate dissolves and infectivity is little affected, but a few hours' exposure to pH 4 at room temperature causes much inactivation. The partitioning of infective and serologically active material between the precipitate and fluid when preparations were centrifuged around pH 4, depended on the time of exposure, the pH value, the virus concentration, salt concentration, and on the amount of extraneous leaf protein present in the preparation. In some conditions, serological activity and infectivity could be almost completely separated, the precipitate containing most of the infectivity, whereas the neutralized soluble material was poorly infective but precipitated strongly with antiserum. Tables 1 and 2 give the results of experiments made in 0·01 M phthalate and show the effects of different times of exposure to acid and of different pH

Table 1. *Effect of acid on ratio of infectivity to serological activity*

(Virus preparation in pH 3·95 0·01 M phthalate buffer and left for specified time before either neutralizing or centrifuging and neutralizing.)

Treatment	Precipitation end-point (mg./l.)	Mean lesions per half-leaf at 100 mg./l.	10 mg./l.	1 mg./l.
Control at pH 7	12	127	32	4
Neutralized after 30 min. at 16°	12	105	17	3
Separated after 15 min. at 16°:				
Neutralized fluid	12	115	31	6
Neutralized precipitate	>100	24	2	0
Separated after 14 hr. at 0°:				
Neutralized fluid	12	72	16	2
Neutralized precipitate	>100	42	12	0·5

Table 2. *Differential separation of infective material on precipitates formed at different* pH *values*

(Virus preparation in 0·01 M phthalate buffer, centrifuged after standing for 15 min. supernatant fluids and resuspended precipitates neutralized before testing.)

pH	Fraction	Precipitation end-point (mg./l.)	Mean lesions per half-leaf at 10 mg./l.	Mean lesions per half-leaf at 1 mg./l.
4·4	Fluid	12	51	10
	Solid	>100	5	0·2
3·9	Fluid	12	56	11
	Solid	>100	31	3
3·5	Fluid	12	11	2
	Solid	>100	68	8

values. In the almost complete absence of salts, for example, using dialysed virus preparations and adjusting the pH with 0·01 M-HCl, and with more concentrated virus preparations, infectivity and serological activity could be separated still more sharply. The material soluble at pH 4 may precipitate with antiserum at concentrations down to 6 mg./l., whereas the acid-precipitable material may precipitate only at concentrations above 25 mg./l., yet the latter may be a hundred times as infective. With virus preparations made so that they contain little normal plant protein, such dramatic dissociations of infectivity from serological activity are unusual, and much of the infective part of the preparation remains soluble between pH 3–4·5. With such preparations, however, the separation can be brought about by the addition of an unstable sedimentable protein that can be isolated from uninfected tobacco leaves (Pirie, 1950). Table 3 records an experiment showing

Table 3. *The removal of infective material by normal plant protein precipitated at* pH 4

(Virus preparation, or virus preparation plus an equal weight of normal leaf protein, in 0·01 M phthalate buffer, centrifuged after 15 min. and precipitate and supernatant fluid neutralized before testing.)

Treatment	Precipitation end-point (mg./l.)	Mean lesions per half-leaf at 100 mg./l.	Mean lesions per half-leaf at 10 mg./l.	Mean lesions per half-leaf at 1 mg./l.
Control at pH 7	12	77	13	4
Preparation at pH 4:				
Fluid	12	67	8	5
Precipitate	100	4	1	0
Preparation plus normal protein	12	51	7	4
Preparation plus normal protein at pH 4:				
Fluid	12	2	0	0
Precipitate	50	49	5	1

the removal of the infective component from a virus preparation on the precipitate of this protein produced at pH 4. In this experiment the simple presence of the normal protein reduced the number of lesions; this was not usually observed and in controls in which the ratio of virus to normal protein was varied in the range 2·5 : 1 to 1 : 50 no systematic influence on the in-

fectivity was found. To get sharp separation, the protein must be freshly prepared and ultracentrifuged sufficiently often to free it from acid-precipitable proteins of low molecular weight, for these prevent the separation of the infective part of a virus preparation when the weights of normal protein and of the virus preparation are approximately equal. When much larger amounts of the normal protein were added, parts of the virus preparation precipitated in acid solution only when the salt concentration was less than 0·02 M. Infective material does not therefore separate on the precipitate that forms when infective sap is acidified, but it does if the sap is first dialysed. Sedimentable proteins from tomato and French-bean leaves can also be used to fractionate the virus preparations; our previous (Bawden & Pirie, 1945*a*) failure to demonstrate this phenomenon probably occurred because the bean protein then used was too old and insufficiently freed from contaminants with low molecular weights. We have added other materials, for example, nucleic acid and hyaluronic acid, to acid solutions of the acid-soluble component of virus preparations, and we have also attempted to separate the components by differential absorption with charcoal, kaolin, kieselguhr, and leaf fibre, but none has behaved like the leaf protein and produced fractions with widely differing ratios of infectivity to serological activity.

The procedure of separating an acid precipitate, either with the help of normal plant protein remaining in the virus preparations or with added normal protein, has obvious potentialities for obtaining material that is, weight for weight, more infective than that separated by differential centrifugation. Most of the infectivity may be concentrated in the one-tenth of the original virus preparation that precipitates together with the normal plant protein, but we have not been able to devise a method whereby these two can subsequently be separated without destroying infectivity. We do not know whether the infective component reacts specifically with the antisera that precipitate the crystalline component. Only when it separates together with an excess of acid-precipitable plant protein have we any reason to consider that it is reasonably free from the crystalline component, and in this condition the neutralized preparations either fail to precipitate with antisera or do so only at concentrations of 100 mg./l. or greater. This is not evidence that free infective virus does not precipitate with the antiserum; it may simply mean that precipitation is prevented by the presence of large amounts of serologically unspecific protein.

Separation from sap of virus in its least modified state

We have already mentioned that the infectivity of purified preparations of this tobacco necrosis virus depends on the initial treatment given to sap, the final product being more infective if the sap is aged or frozen before it is ultracentrifuged than if it is centrifuged when freshly expressed. It seems from this that the preparations we have so far described are not only demonstrably inhomogeneous, but also contain material that is artefact, derived from some precursor by changes brought about during the treatment. It may well be that some of the components of these preparations occur in the more necrotic

parts of an infected leaf but it would seem reasonable to assume that the state of the virus in infected cells adjacent to necrotic areas more nearly resembles its state in fresh sap. Knowledge of the properties of virus in these cells, rather than in the necrotic ones, is likely to shed light on the physiology of virus multiplication. Attempts have therefore been made to isolate the virus in its least modified form by working with sap kept cold and making fractionations with as little delay as possible.

The sap from freshly minced leaves is expressed into a cylinder cooled with ice and is immediately clarified by centrifuging at 7000 r.p.m.; the supernatant fluid is centrifuged for 30 min. at 40,000 r.p.m. in a rotor chilled with ice. The pellets are evenly suspended in water, using about one-tenth the volume of the original sap, left for an hour at 0° and then centrifuged for 10 min. at 7000 r.p.m. The residue is extracted a second time with water, and the pooled clarified extracts are ultracentrifuged again in a chilled rotor. The pellets are suspended in water and freed from insoluble material at 7000 r.p.m. A third ultracentrifugation may give a further fractionation, but it usually does not do so when the supernatant fluids have been thoroughly drained away after the first two runs. Such preparations are more opalescent than those from frozen sap and they are less stable. After 5–10 days at 3°, about a third of their dry matter coagulates and can be removed by low-speed centrifugation. The coagulation is promoted by the presence of chloroform as an antiseptic and is delayed by 1 g./l. azide; it is not affected by toluene and thymol.

Table 4. *Yields and activities of virus preparations made from sap receiving different initial treatments*

Treatment of sap	Yield (mg./l.)	Precipitation end-point (mg./l.)	Mean lesions per half-leaf at 2 mg./l.	Mean lesions per half-leaf at 0·2 mg./l.
Centrifuged immediately	350	15	17	2
Centrifuged after 2 days at 3°	320	15	133	23
Centrifuged after freezing	180	12	156	40

Table 4 shows the yields, precipitation end-points and relative infectivities of preparations made from samples of the same batch of sap by the method described in the previous paragraph, by the same method but using sap that was left at 3° for 48 hr., and by the method described earlier in this paper. The increase in infectivity, without any commensurate decrease in yield, obtained by the second and third methods is obvious. Increases of this magnitude are achieved only with certain leaves, and the increase in activity produced by ageing sap depends on the age of leaf, the duration of infection and the extent to which the lesions cover the leaf surface. With leaves infected for the same lengths of time, the increase in activity is greatest with those that are yellowest and most completely covered with lesions. This is illustrated by Table 5, which shows the results of an experiment in which leaves from one batch of plants were separated into three groups showing different grades of chlorosis and necrosis. Sap from each group was treated in two ways; one lot was chilled and ultracentrifuged immediately and the other was aged for

Table 5. *Differential activation of virus by ageing in sap from different leaves*

Leaves	Sap	Precipitation end-point (mg./l.)	Mean lesions per half-leaf at 1/1*	1/10*
Youngest, crisp and green	Fresh	25	92	17
	Aged	12	47	5
Intermediate	Fresh	18	67	18
	Aged	6	91	24
Oldest, limp and chlorotic	Fresh	12	88	17
	Aged	6	105	26

* The preparations made from the fresh lots of sap were tested in infectivity tests at four times the concentration of those from the aged ones; actual concentrations were 4 and 0·4 mg./l. for the fresh lots and 1 and 0·1 mg./l. for the aged. The ageing was for 2 days in sap at 3° before ultracentrifugation.

48 hr. at 3° before being ultracentrifuged. The yields of purified virus from the three lots of aged saps were similar, and with all three kinds of leaves larger yields, with lower precipitation end-points, were obtained from the fresh than from the aged samples. All the preparations made from fresh lots of sap had similar infectivities, but those from the aged saps differed. From the most yellowed and from the intermediate leaves, the virus from aged sap was more than four times as infective as that from fresh sap, whereas ageing had little effect on the infectivity of the virus from the greenest leaves. A further portion of sap from the yellowest leaves was frozen before being ultracentrifuged and the virus prepared from this, as in the experiment shown in Table 4, was still more infective than that prepared from aged sap. Experiments were also made in which batches of leaves from comparable positions were picked from one set of infected plants at 4, 7 and 10 days after inoculation. All the samples of virus prepared immediately from chilled sap were again about equally infective, whereas the samples prepared from sap allowed to age for 48 hr. at 3° differed, those from the leaves taken soonest after inoculation being less infective than the later ones. Here again the difference lies, not in the virus as it is first obtained from the leaves, but in the conditions obtaining in sap; sap from extensively necrotic leaves gave increased infectivity on ageing and that from other leaves failing to do so.

Although we can gauge from the appearance of infected leaves whether or not ageing in sap will increase infectivity, we do not know the processes involved. Ultracentrifuged unactivated virus has been exposed to various salt solutions and to sap, both fresh and aged, from old and young uninfected leaves, and to the supernatant fluid from ultracentrifuged infective sap, but none of these treatments has produced unequivocal activation. Because of this failure, it has been possible to study the mechanism of activation only in infective sap. At 3° maximum infectivity is reached after 2–4 days, and infectivity then gradually declines; at room temperature, inactivation is so much more rapid that the initial activation may be obscured. Oxygen does not seem to be needed, for infectivity increased in samples of sap from which

the air had been largely removed by exposure to vacuum. Freezing for 1–5 hr. at −5 to −10° caused activation, and prolonged freezing did not decrease infectivity. When sap is dialysed, a large precipitate separates and virus prepared from the supernatant fluid is sometimes more and sometimes less infective than that prepared from fresh sap. Factors that we are not controlling probably affect the extent to which infective particles are adsorbed by this precipitate. Heating sap at 30° for short periods, and ageing pellets ultracentrifuged from fresh sap in their supernatant fluids at 3°, have also sometimes increased infectivity. These phenomena are illustrated by the experiment recorded in Table 6, but they do not always occur and we cannot explain the frequent failures we have had in experiments intended to serve as repetitions of this one.

Table 6. *Effects of different treatments in increasing infectivity*

Treatment	Mean lesions per half-leaf at 5 mg./l.	Mean lesions per half-leaf at 0·5 mg./l.
Virus from fresh sap	8	1·5
Above at double concentration	15	2·5
Pellet from fresh sap exposed to supernatant fluid	22·5	4·2
Sap heated for 4 hr. at 27°	29	4
Sap aged for 2 days at 3°	26	6

After being subjected to the specified treatment, the virus was twice sedimented at 40,000 r.p.m. and resuspended in water before testing.

The treatments that increase the infectivity of virus in sap all precipitate some of the normal protein and polysaccharide, suggesting that some component of this mixture may inhibit infectivity and that its removal explains the phenomenon. The precipitation proceeds spontaneously even at 3° but it can be diminished by adjusting the pH to 6·5 or 7·5, or by adding 1 g./l. of sodium azide, but these treatments do not prevent the activation. Apparently similar precipitates also separate from samples of sap in which ageing at 3° produces little or no increase in infectivity. Precipitation, therefore, is probably not significant. Most of the components that separate during ageing can be precipitated from sap at pH 4. Because of the salt content of sap, little infective or serologically active material separates with this precipitate, and the treatment usually increases the infectivity of the supernatant fluid, but always to a smaller extent than ageing or freezing. We have already mentioned that precipitates separate from virus preparations made from fresh sap, but this produces no greater increase in infectivity than would be expected from the removal of contaminating inert material and it leaves the ratio between infectivity and serological activity unaffected. It seems unlikely that the difference between activated and other preparations can be explained by the presence of inhibitors in the latter which are destroyed during the processes of activation. When small quantities of fresh tobacco sap, or the sedimentable components from it, are added to activated virus there is no striking reduction

in infectivity, and when the virus has been quickly re-isolated from mixtures with fresh sap by ultracentrifugation in the cold, it has been recovered with its full infectivity.

Properties of activated and unactivated virus preparations

Unactivated preparations made by isolating the virus from fresh cold sap contain some sedimentable normal leaf protein. This can be removed by precipitation at pH 4, when, as already stated, it usually carries with it most of the infective part of the virus preparation. This protein denatures in a few days at room temperature in the presence of chloroform or in a longer period at 0°. The infectivity of the fluid is unaltered by this precipitation. If there is any activation, it is compensated by an equal removal of infective material on the precipitate. The coagulation of the normal protein is associated with the production of low molecular weight components, which can then be separated from the virus by a further ultracentrifugation. When this has been done, comparisons between such preparations and the more infective ones made from aged or frozen sap have revealed no significant or systematic differences in chemical and physical properties. When they are centrifuged at 20,000 r.p.m., both kinds of preparation separate into pellets and uncompacted material, and with both the material in the pellet is, weight for weight, more infective. Thus, there are no gross differences in particle weights between the components occurring in the two kinds of preparation.

All preparations made by the methods described in this paper have had higher carbohydrate contents than those described in 1945. Then, 7–8·5 % was the usual range, and this is what we now find for preparations that are sedimented again after lying for some weeks at 3°. The carbohydrate content of fresh preparations varies from 14 to 22 %; the phosphorus content varies between 1·7 and 2·0 %, as in our earlier preparations. Fresh preparations, therefore, do not contain all their carbohydrate in the form of a nucleic acid. We (Bawden & Pirie, 1938*a*, *b*) found extra carbohydrate associated with incompletely purified preparations of potato virus *X* and tomato bushy stunt virus, but it seemed most probable that this was simply mixed with these viruses. The fact that the carbohydrate in preparations of the Rothamsted tobacco necrosis virus becomes unsedimentable after a time suggests that, in fresh preparations, it is combined with the virus but that the linkage is labile. The breaking of this linkage may control the susceptibility of the virus to the inactivation produced by exposure to citrate, which is described in the accompanying paper (Bawden & Pirie, 1950). Sufficient material has not yet been obtained for a thorough study of the carbohydrate that occurs in the supernatant fluid when old virus preparations are centrifuged.

When examined in the electron microscope even preparations that have been fractionated by sedimentation at 20,000 r.p.m. contain particles of two sizes, one about 37 mμ and the other about 17 mμ in diameter; these are presumably the components previously described that give sedimentation constants of 235 and 49 (Ogston, 1942). The smaller particles appear to be spherical but the larger, after drying and metal shadowing, have an apparently mamillated

surface compatible with the idea that they are built up by the coalescence of smaller particles. Preparations that consist exclusively of the small particles can be made from the crystals that separate slowly from concentrated virus solutions in water, and the particles in them have a tendency to adhere regularly in sheets similar to those found by Wyckoff (1949) in the crystals themselves. These preparations are almost devoid of infectivity. Preparations consisting almost exclusively of the larger particles may also be of low infectivity. Comparisons have been made between quickly prepared unactivated preparations and aged activated preparations from the same batch of sap. The ratio of large to small particles in these may be the same. This is in agreement with the similar behaviour of the two types of preparation when fractionated ultracentrifugally. The fact that the more readily sedimenting fraction is the more infective naturally suggests that the larger particles are the vehicle of infectivity, but it is clear that not all of them carry infectivity and that the difference between an infective and a non-infective particle is not at present perceptible on an electron-microgram. We hope to deal more thoroughly with these relationships in a later paper.

Release of virus from the residues of infected leaves

Tomato bushy stunt and tobacco mosaic viruses can be extracted in large amounts from the fibrous residues remaining after infected leaves have been minced and squeezed to express the sap, either by fine grinding or by digesting the residues with enzymes from the crop of the snail (Bawden & Pirie, 1944, 1945*b*). These treatments also liberate, from leaves infected with the Rothamsted tobacco necrosis virus, material that reacts specifically with the virus antiserum. Fine grinding disperses normal leaf components into a form in which they are not readily separated from the virus preparations by ultracentrifugation, and the incubation necessary for liberation by the snail enzymes in itself largely destroys infectivity. Hence these methods are not suitable for studying the infectivity of the virus that remains in the leaf residues. Of the other methods we have tried, only simple washing will be discussed.

When fibre that has been minced twice and pressed by hand is suspended in water, additional material that reacts with virus antiserum is extracted. Extraction is slow and the precipitin titre of the extracts increases for periods of extraction up to 2 days. The total yield of serologically active material that can be extracted in this manner and recovered by sedimentation from the extracts is about half that prepared from the sap from the same sample of fibre. The yield is slightly increased by additional passages of the fibre through a mincer. The nature of the extracting fluid has little effect on the yield; thus the precipitin titres of extracts made with water, 20 g./l. or 2 g./l. sodium chloride, or with the supernatant fluid from ultracentrifuged infective sap, have not differed by a factor of two. The infectivities of extracts in the different fluids have, on the other hand, differed greatly. The interpretation of these differences is complicated by our ignorance of the extent to which differences arise from different abilities of the fluids to extract different amounts of the

infective particles, or from their differing abilities to affect infectivity after the virus has been extracted from the fibre. Successive extracts with water give material that shows a progressively smaller ratio of infectivity to serological activity. This phenomenon is illustrated by the experiment given in Table 7. The successive extracts were made with diminishing volumes of water,

Table 7. *Infectivity of material obtained from minced leaves in successive extracts*

Extract	Antigen content (precipitation end-point × volume in ml.)	Mean lesions per half-leaf at 1/1*	1/10*
Sap	1860	115	46
1st water	320	85	52
2nd water	200	44	14
3rd water	160	12	2
4th water	150	4	2
5th water	100	4	0·2

Infectivity tests were made with all fluids diluted to equal antigen contents, these were approximately 1/1 = 5 mg./l. and 1/10 = 0·5 mg./l.

so that the virus contents of the extracts should not differ too widely. The virus in each extract was concentrated by ultracentrifugation, the precipitin titres were determined and then infectivity tests were made at dilutions corresponding to equal antigen contents. It can be seen that, after the first extract, in which residual sap was present, the infectivity fell sharply.

This phenomenon again does not seem to be explicable by postulating that inhibitors of infectivity are extracted in increasing amounts in successive extracts, for when the virus from sap, from the first and from successive extracts, has been purified by repeated ultracentrifugations, the differences in infectivity have persisted unchanged. When salt solutions are used as extractants, the ratio of infectivity to serological activity in successive extracts differs less than with water, and using the supernatant fluid from ultracentrifuged infective sap, there may be no difference. This is shown in Table 8. This experiment was done with the fibrous residues from the leaves used for the

Table 8. *Relative infectivities of virus obtained in successive extracts from leaves of different ages*

	Mean lesions per half-leaf from virus in					
	Sap		Residue extracted with water		Residue extracted with supernatant fluid	
Leaves	1/1	1/10	1/1	1/10	1/1	1/10
Young, green	114	51	21	4	74	30
Intermediate	70	29	43	9	74	29
Old, yellow	126	45	122	31	146	60

Infectivity tests were made with all fluids diluted to equal antigen contents, these were approximately 1/1 = 5 mg./l. and 1/10 = 0·5 mg./l.

experiment given in Table 5, and the residues were extracted successively with water and the supernatant fluid. The virus in each extract was concentrated by ultracentrifugation, precipitin titres were determined and infectivity tests made at comparable antigen contents. The young and intermediate groups of leaves show clearly the failure of water to give extracts as infective as those subsequently obtained from the same fibre with clarified sap. There was no such phenomenon with the yellowest leaves, sap from which gave the greatest activation on ageing, and this suggests that the phenomenon may result from the failure of activation to occur in water, rather than from a differential extraction of already infective particles into the different ionic environments. This idea receives some further support from the fact that fibre from healthy plants does not preferentially adsorb infective particles from virus preparations whether in water or in sap.

DISCUSSION

Our results do not simplify knowledge of viruses; rather the reverse. They show that changes in infectivity may occur in the initial extract and in fluids that are usually regarded as bland; they seem inexplicable on the basis of a single unit particle in which changes can only cause loss of infectivity. Leaf sap, made by mincing fresh leaves and pressing lightly, is an environment in which this tobacco necrosis virus does not maintain its properties constant. In this respect it is not unique, for many others lose infectivity rapidly in sap, and tobacco mosaic virus, which retains its infectivity for long periods in sap, changes its physical state (Bawden & Pirie, 1945*b*). The tobacco necrosis virus is unusual in that, in addition to changes that decrease infectivity, others are demonstrable that increase it. Also unusual is the occurrence in infective sap of at least two kinds of particles differing in size, neither of which occurs in sap from healthy leaves. Turnip yellow mosiac virus may be analogous, for sap from infected plants contains two specific particles that differ in chemical constitution but have similar sizes (Markham & Smith, 1949). So many variables influence the activity of the Rothamsted tobacco necrosis virus that, even in freshly expressed sap, it may not necessarily be in the form in which it occurs in intact cells, nor may it have the activities it has there. Sap is not a physiological fluid and viruses are not exposed to it until leaves are minced. There is no *a priori* reason why it should be suitable for maintaining viruses with their original properties, and it may be that the properties of preparations of other viruses made without precautions designed to prevent changes are also not those of the viruses in their original habitats. This is not a plea for limiting work to unfractionated extracts; that would not solve the problem, because as soon as cells are damaged changes begin. These may be unavoidable, but an awareness of them should not lead to a vitalist defeatism about the possibility of gaining information about the intrinsic properties of viruses. It should simply lead to caution in attributing the properties of purified material to the virus in the cell.

Ultimately the components of the leaf that cause changes in the extracted virus may be removed without exposing the virus to them, or it may be

possible to infiltrate substances into intact leaves that will protect the virus from change. At present the best that can be done is to release the virus into sap at a low temperature and then isolate it as rapidly as possible. This obviously does not ensure that changes have been avoided, but there will most likely be less change and the virus will be more nearly in its original state than when prepared by more dilatory methods. If this assumption is justified, then a possible, although extreme, interpretation of the increased infectivity caused by ageing in sap, or by freezing sap, is that undamaged cells contain no virus particles capable of causing infection and that infectivity is a property acquired only when particles are liberated into a suitable medium. A similar change might occur without experimental intervention in necrotic and withered parts of leaves, but infectivity may not be a property of any particles in undamaged cells.

We have called the preparations of materials sedimented from infective sap, virus preparations, but it is uncertain which of the components of the preparations most warrants the name virus. There appear to be at least four substances in the preparations of sedimentable material we have made from extracts of infected leaves:

A. The crystallizable nucleoprotein that we have described earlier (Bawden & Pirie, 1945*a*). This appears to be the antigen specific to infected plants and it occurs in species as remotely related as tobacco, French bean and tulip when they are infected with the Rothamsted tobacco necrosis virus. It is the major constituent of most purified preparations but we have no evidence that it is ever infective. If it is, infectivity is a transient phase, for by the time homogeneous preparations are obtained infectivity is almost wholly gone.

B. The infective material that is made by freezing or ageing in sap; this sediments more rapidly than A.

C. The precursor of B, which is either less infective than B or is wholly non-infective.

D. Unstable normal leaf protein.

The only reason we have for postulating a relationship between A and B is that antisera made against non-infective preparations of A specifically neutralize the infectivity of preparations of the virus (Kassanis, 1943). Its occurrence in infected hosts of unrelated species, and its absence from plants showing identical symptoms caused by other tobacco necrosis viruses, suggest that it is a product specific to the activities of the Rothamsted virus and not simply a consequence of necrosis. It may be a stage in the development of virus particles, but this is not established and there is nothing to suggest whether it is more likely to be a stage in synthesis or degeneration. Antisera made against substance A precipitate infective preparations, but it is unknown whether this is because they combine with substance B. Our most infective preparations give lower precipitation end-points than do preparations of substance A, suggesting that precipitation may occur because of contamination with substance A. Pellets centrifuged from the most infective preparations contain some scattered crystals and electron-micrograms also show the presence of particles about 17 mμ. in diameter mixed with larger ones. This is

evidence of contamination with substance A, but A and B may be serologically related and the lower precipitin end-points of infective preparations may merely be a result of the larger particle size of B.

The relationships between B and C are wholly unknown, except that differential centrifugation and electron microscopy suggest that they do not differ widely in size. An activation of the type found might occur from the addition, removal, or rearrangement of groups, and we have no observations to favour one possibility rather than another. Substance D is a contaminant of all freshly made preparations, but occurs especially in those made without delay from chilled sap. Only traces occur in preparations that have been frozen or have been sedimented again after lying for some weeks. The only connexion of which we are aware that it has with the virus is its ability, when fresh, to precipitate component B to a greater extent than A at pH 4.

Our results are most simply explained by the assumption that D is an irrelevant contaminant, that B and C are aggregates containing approximately the same number of particles physically comparable to A. The mechanism that brings about the transition between C and B is of great interest, but much valuable information will be lost if attention is exclusively directed towards infective components. At many stages in the development of symptoms the greatest part of the anomalous nucleoprotein of the leaf appears to be in non-infective states and any picture of the development of a virus disease must include them.

If phenomena similar to those described here are at all widespread, it is clearly dangerous to assume that any anomalous material that can be ultra-centrifuged from an infective extract, or any anomalous particle that can be seen in an electron-microgram, is an entity capable of infecting a new host. We have already described the separation of several different nucleoproteins from the sap of plants infected with tobacco mosaic virus and it seems probable that other viruses, if studied intensively, will be found to be associated with a comparable complex range of substances.

We wish to thank Mr H. L. Nixon for examining the preparations with the electron microscope.

REFERENCES

Bawden, F. C. (1941). The serological reactions of viruses causing tobacco necrosis. *Brit. J. exp. Path.* **22**, 59.

Bawden, F. C. & Pirie, N. W. (1937). The isolation and some properties of liquid crystalline substances from solanaceous plants infected with three strains of tobacco mosaic virus. *Proc. roy. Soc.* B, **123**, 274.

Bawden, F. C. & Pirie, N. W. (1938*a*). Liquid crystalline preparations of potato virus *X*. *Brit. J. exp. Path.* **19**, 66.

Bawden, F. C. & Pirie, N. W. (1938*b*). Crystalline preparations of tomato bushy stunt virus. *Brit. J. exp. Path.* **19**, 251.

Bawden, F. C. & Pirie, N. W. (1942). A preliminary description of preparations of some of the viruses causing tobacco necrosis. *Brit. J. exp. Path.* **23**, 314.

Bawden, F. C. & Pirie, N. W. (1944). The liberation of virus, together with materials that inhibit its precipitation with antiserum, from the solid leaf residues of tomato plants suffering from bushy stunt. *Brit. J. exp. Path.* **25**, 68.

Bawden, F. C. & Pirie, N. W. (1945*a*). Further studies on the purification and properties of a virus causing tobacco necrosis. *Brit. J. exp. Path.* **26**, 277.

Bawden, F. C. & Pirie, N. W. (1945*b*). The separation and properties of tobacco mosaic virus in different states of aggregation. *Brit. J. exp. Path.* **26**, 294.

Bawden, F. C. & Pirie, N. W. (1950). Some effects of freezing in the leaf, and of citrate *in vitro*, on the infectivity of a tobacco necrosis virus. *J. gen. Microbiol.* **4**, 482.

Bawden, F. C. & Roberts, F. M. (1947). The influence of light intensity on the susceptibility of plants to certain viruses. *Ann. appl. Biol.* **34**, 286.

Bawden, F. C. & Roberts, F. M. (1948). Photosynthesis and predisposition of plants to infection with certain viruses. *Ann. appl. Biol.* **35**, 418.

Boyle, R. (1683). *New experiments and observations touching cold.*

Glauber, J. R. (1658). *Miraculi Mundi Continuatio*, p. 215. *Opera Chymica*, Franckfurt.

Kassanis, B. (1943). Neutralization of some plant viruses by rabbit sera. *Brit. J. exp. Path.* **24**, 152.

Markham, R. & Smith, K. M. (1949). Studies on the virus of turnip yellow mosaic. *Parasitology*, **39**, 330.

Masket, A. V. (1941). A quantity type rotor for the ultracentrifuge. *Rev. sci. Instrum.* **12**, 277.

Mellanby, J. (1908). Diphtheria antitoxin. *Proc. roy. Soc.* B, **80**, 399.

Ogston, A. G. (1942). Examination in the ultracentrifuge. *Brit. J. exp. Path.* **23**, 328.

Palmer, A. H. (1934). The preparation of a crystalline globulin from the albumin fraction of cow's milk. *J. biol. Chem.* **104**, 359.

Paterson, J. S., Pirie, N. W. & Stableforth, A. W. (1947). Protective antigens isolated from *Br. abortus*. *Brit. J. exp. Path.* **28**, 223.

Pirie, N. W. (1950). The isolation, from normal tobacco leaves, of nucleoprotein with some similarity to plant viruses. *Biochem. J.* (In the Press.)

Smith, K. M. & Bald, J. G. (1935). A description of a necrotic virus disease affecting tobacco and other plants. *Parasitology*, **27**, 231.

Veer, G. de (1596). *The third voyage Northward to the Kingdoms of Cathaia, and China, in Anno* 1596. Quoted from (1906) *Purchas his Pilgrimes*, **13**, 91. Glasgow: MacLehose and Sons.

Wyckoff, R. W. G. (1949). *Electron microscopy.* New York: Interscience Publishers.

(*Received* 25 *February* 1950)

Some Effects of Freezing in the Leaf, and of Citrate *in vitro*, on the Infectivity of a Tobacco Necrosis Virus

By F. C. BAWDEN and N. W. PIRIE

Rothamsted Experimental Station, Harpenden, Hertfordshire

SUMMARY: Preparations of the Rothamsted tobacco necrosis virus made from tobacco leaves that have been frozen while intact are less infective than preparations made from unfrozen leaves. Freezing minced leaves or expressed sap does not destroy infectivity. The suggestion is made that much virus in the intact leaf becomes infective only by means of a mechanism that is set in action by mincing and is disordered by freezing.

The infectivity, but not the serological activity, of the virus is lost on exposure to 0·02 0·01M neutral citrate; the extent of this inactivation is influenced by the temperature, pH, duration of exposure, concentration of virus and presence of salts and other substances. Similar processes could influence the infectivity of the virus in sap and may do so in the leaf.

The sensitivity of the Rothamsted culture of a tobacco necrosis virus towards changes in the composition of the fluid in which it is dissolved separates it sharply from tomato bushy stunt or tobacco mosaic viruses. In fluids having compositions comparable to those derived from macerated leaves, differences in infectivity with the last two viruses are usually attributed to the presence of varying amounts of substances that interfere with the normal processes of infection. Such inhibitors have no permanent effects on the viruses, which regain their initial infectivity when the substances are removed. By contrast, the infectivity of the Rothamsted tobacco necrosis virus can be increased or decreased by exposure to environments that appear not to influence the infectivity of the other two. In particular, infectivity does not remain constant when the virus is in the sap obtained by mincing and lightly pressing infected tobacco leaves. The differences persist through subsequent stages of isolation and the infectivity of a purified preparation depends on the physiological state of the plants from which it came and on the method of preparation used (Bawden & Pirie, 1950).

The Rothamsted tobacco necrosis virus loses infectivity when exposed to some substances with low molecular weight. In an attempt to avoid exposing it to the low molecular weight components of sap, we subjected infected leaves to various treatments that liberate these components but not the virus. Although these treatments were innocuous to the isolated virus, or to the virus in minced leaves, when applied to intact leaves they decreased the infectivity, or the total yield, or both. This paper describes treatments that inactivate virus in the leaves and some of the variables that influence the inactivation of the isolated virus. The results of these experiments show that so many factors can affect the infectivity of this virus that we seem, in effects of this type, to have a partial explanation of the wide variations found in the properties of different preparations of the virus. There is no evidence about the

state in which the virus occurs in infected cells, nor is there evidence that the actual phenomena described have any significance in the processes of infection and virus multiplication. The object of this paper is to direct attention to phenomena that may play a part in these processes, and also perhaps help to determine the resistance of plants to viruses, in the hope that later work may uncover the precise mechanisms involved.

MATERIALS AND METHODS

The virus was propagated in tobacco plants (*Nicotiana tabacum* var. White Burley), raised under low light intensity and placed in darkness for 1–2 days before they were inoculated. The leaves were lightly dusted with celite before being rubbed with infective sap. Leaves were picked from 5 to 8 days after being rubbed, before the lesions had coalesced and caused the tissue to dry unduly, and were minced by passage through a domestic meat mincer.

Virus was isolated from clarified sap by ultracentrifugation as already described (Bawden & Pirie, 1950). Unless otherwise stated, the virus in sap was concentrated by freezing or the sap was allowed to stand at 3° for 2 days before the first ultracentrifugation. The purified virus preparations were kept in water at about pH 6, an environment in which infectivity changed little during 1–2 weeks' storage at 3°. However, other changes did occur and these may cause some of the variability of our results.

Antisera were prepared by intravenous injections of purified virus into rabbits, and precipitin tests were made by adding 1 ml. of virus preparations at various dilutions to each of a series of tubes containing 1 ml. of antiserum at 1/50. Precipitin titres were taken as the greatest dilution, or, when purified material was used, the smallest concentration, of antigen to give a precipitate visible to the eye after 3 hr. incubation in a water-bath at 50°. Serial dilutions were usually made with a factor of two, but by observing precipitation at intervals and judging from the time taken for precipitation to occur at different dilutions and from the size of the precipitates, differences of less than this factor were estimated with fair accuracy.

Infectivity tests were made on French bean, *Phaseolus vulgaris* var. Prince, using the first formed leaves. Usually six or four inocula were compared together in any one test, and the different inocula were distributed over half-leaves selected so that each occurred an equal number of times on left- and right-hand halves and all pairs occurred on the same number of leaves. Tests were made at two dilutions of inoculum, usually varying by a factor of ten, and there were at least six replications at each dilution. The variations between the results of experiments made at different times are not thought to result from errors in infectivity tests. When the same experiment has been repeated after a short interval, reproducible results have been obtained. Variations occurred mainly when comparing effects of similar treatments on different preparations of the virus. As the susceptibility of bean leaves is enhanced by placing the plants in darkness before they are inoculated (Bawden & Roberts, 1947, 1948), it seemed possible that some variations might arise from making assays for infectivity at different times of the day. Control experiments,

however, excluded this as an important factor, for although the total number of lesions produced by a given inoculum was affected, there was no appreciable effect on the relative numbers produced by different inocula. Other experiments also showed that no appreciable variability arose within a few hours from differences in time that elapsed between making test dilutions and applying the inocula to bean leaves. The time at which lesions are counted is of some importance when the inocula being compared produce widely different numbers. In the interval between the earliest and latest times at which lesions can be counted at all accurately, leaves with few lesions produce proportionally more additional ones than leaves with many. The effect is too small to explain variations observed between experiments with different virus preparations, but it was avoided, as far as possible, by delaying counting until the first-formed lesions began to spread along the veins.

EXPERIMENTAL

Inactivation by treatments applied to the intact leaf

Variations in the composition of leaf extracts may influence the number of lesions produced by a given quantity of infective virus. To diminish this source of error, in all experiments with virus from variously treated leaves, the extracts were ultracentrifuged and the pellet, resuspended in water, was used for comparative infectivity tests. In the type of centrifuge used (Masket, 1941), much of the pellet may redissolve in the bottom 0·5 ml. of supernatant fluid, particularly if the virus content is small or the extracts have been subjected to any pre-treatments that reduce the amount of sedimentable material. After centrifuging for 30 min. at 40,000 r.p.m., therefore, the supernatant fluids were not poured off, but were carefully siphoned away leaving the bottom 0·5 ml. The pellet was extracted with this residual fluid plus 1·5 ml. of water, and after standing for an hour was centrifuged for a few minutes at 10,000 *g* to remove insoluble material.

Table 1 shows the results of one experiment in which similar lots of infected leaves from the same batch were treated in four different ways. Freezing the intact leaves before they were minced decreased the infectivity of the preparation without correspondingly decreasing its serological activity. By contrast, freezing the expressed sap had little effect on either of these specific properties, although sap stored at 3° for 24 hr. had the greatest ratio of infectivity to serological activity—a result of the activation in sap already

Table 1. *Effect of freezing on tobacco necrosis virus in sap and in intact leaves*

Treatment	Precipitin titre	Infectivity Mean lesions per half-leaf at 1/50	1/500
Sap, 24 hr. at 3°	1/48	86	22
Sap, 24 hr. at −10°	1/32	85	14
Intact leaves, 24 hr. at 3°	1/32	84	11
Intact leaves, 24 hr. at −10°	1/32	18	1

described (Bawden & Pirie, 1950). It was only when intact leaves were frozen that the ratio of infectivity to serological activity was decreased, and freezing minced leaves before pressing out the sap, like freezing the expressed sap, did not decrease it. The phenomenon occurs regularly when intact leaves are frozen, though the magnitude of the effect has varied somewhat with different lots of leaves. Durations of freezing between 3 and 48 hr. had no consistent effect, but the treatment of the leaves between freezing and mincing did influence the properties of the extractable material. Table 2 compares the

Table 2. *Effect of freezing and washing intact leaves on ratio of serological activity and infectivity*

Extract from	Precipitin titre	Infectivity Mean lesions per half-leaf at 1/20	1/100	1/500
Unfrozen leaves	1/24	85	48	24
Frozen leaves, thawed before mincing	1/8	28	9	2
Frozen leaves, washed once before mincing	1/8	12	4	1
Frozen leaves, washed twice before mincing	1/8	6	1	0

infectivity and serological activity of extracts from samples of one batch of leaves treated in different ways: unfrozen; frozen, thawed and minced without washing; and frozen and washed by stirring gently once or twice in twenty times their weight of distilled water. The diminution in infectivity caused by freezing is again obvious, and the decrease in the ratio of infectivity to serological activity was progressively increased by washing the leaves before mincing. As with tobacco mosaic virus (Bawden & Pirie, 1945*b*), the washes contained little or no virus, whether this was assayed by infectivity tests or serologically. Freezing had not apparently anchored the infective virus to the leaf residue in such a way that it could be separated again by extraction with a suitable solvent. Many unsuccessful attempts were made to extract infective material from the leaf residues that remained after squeezing sap from minced leaves that had been frozen. Sap from uninfected tobacco leaves, the supernatant fluid from infective sap centrifuged at 40,000 r.p.m., the fluid that could be pressed from thawed unminced leaves, and various concentrations of sodium chloride and sodium phosphate, were all tried, but none extracted material with sufficient infectivity to account for that lost by freezing the intact leaves. The possibility that freezing extracted an inhibitor which accompanies the virus during one sedimentation was rendered unlikely by experiments in which virus from frozen and unfrozen leaves was further purified. One such comparison is shown in Table 3, from which it will be seen that, after three sedimentations, the material from frozen leaves had a rather greater serological activity than that from unfrozen leaves, but much less infectivity. Freezing either intact leaves or sap denatures many normal plant proteins and facilitates the preparation of the virus in the form in which it gives crystalline pellets and high serological titres.

Table 3. *The yields and relative activities of virus preparations made from frozen and unfrozen leaves*

Preparation	Yield (mg./l.)	Precipitation end-point in mg./l.	Infectivity Mean lesions per half-leaf at 10 mg./l.	Infectivity Mean lesions per half-leaf at 1 mg./l.
From frozen leaves	170	12	11	1·5
From unfrozen leaves	200	18	42	6·5

Experiments comparable to those with frozen leaves were made by impairing the osmotic control of leaves by exposing them to chloroform or toluene, or by infiltrating water by repeatedly exposing immersed leaves to vacuum. In several experiments intact leaves were exposed to air saturated with chloroform for 1–3 days. The now limp and sodden leaves were then minced, the sap expressed and ultracentrifuged. The resulting extracts, like those from frozen leaves, were clearer than those from fresh leaves, and they sometimes gave a crystalline pellet on the first ultracentrifugation. Their properties have varied. Sometimes the material was much less infective than that sedimented from minced fresh leaves or from those kept intact for a comparable time without exposure to chloroform, but more often the total yield of virus was decreased and that extracted had about the same ratio of infectivity to serological activity as control pellets. The variations suggest that the treatments were causing protein destruction within the leaf cells and that the effect produced depended on the physiological condition of the leaves; we have made no attempt to control this more closely.

Inactivation in vitro *by citrate*

The readiness with which changes in the technique of preparation affect the infectivity of purified preparations of the Rothamsted tobacco necrosis virus (Bawden & Pirie, 1950) suggested that the virus was sensitive to variations occurring in sap, particularly as previous work (Bawden & Pirie, 1945*a*) had shown that exposure to moderate concentrations of sodium chloride and phosphate caused inactivation. We therefore studied the effect of several substances at concentrations within the range normal for leaves, in the hope of gaining information that might throw some light on the changes that proceed in infected leaves and sap. It soon became obvious that the extent of inactivation caused by different agents depended on so many variables that an extensive study of different agents was impossible, and we have restricted our experiments to the citrate ion. Even so, it has not yet proved possible so to define conditions that results are precisely reproducible after an interval. This may be because of changes continuously occurring in the purified virus preparations that affect their inactivation by citrate, and our experiments with preparations of different ages support this possibility. However, as every variable we have attempted to control has influenced the extent to which citrate inactivates, there are almost certainly others that do so and that we have not attempted to control.

Fig. 1 shows the course of inactivation of two virus preparations at 18° in 0·02 M sodium citrate (pH 5·8). Equal volumes of 0·04 M sodium citrate and 1·5 g./l. virus were mixed at suitable times; all were diluted at the same time and compared for their infectivity with samples of the virus preparations to which citrate was added after dilution and immediately before inoculation to test plants. The final virus concentrations in the inocula were 5 and 0·5 mg./l.,

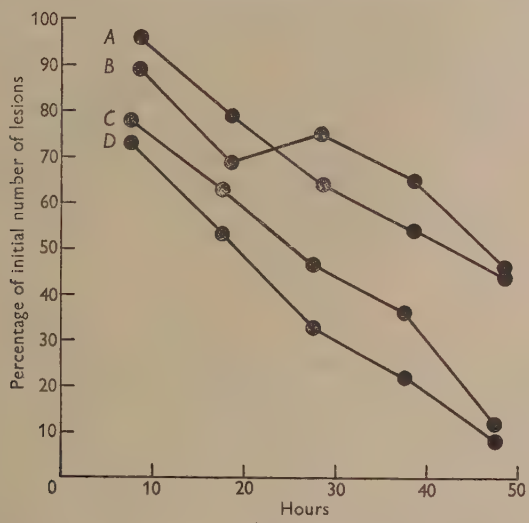


Fig. 1. Virus at 0·75 g./l. was exposed to 0·02 M citrate (pH 5·8) at 18° for various times and then tested on beans at two dilutions. The number of lesions after different periods of exposure is expressed as a percentage of the number given by a sample to which citrate was added after dilution and immediately before test; this number (mean per half-leaf) for each experiment was: *A*, freshly prepared virus tested at 5 mg./l., 126; *B*, freshly prepared virus tested at 0·5 mg./l., 45; *C*, older virus tested at 5 mg./l., 115; *D*, older virus tested at 0·5 mg./l., 39.

and the results are recorded by expressing the numbers of lesions produced by the samples exposed to citrate for various lengths of time as percentages of the numbers produced by the relevant control, which was exposed only momentarily to citrate diluted 1/150. When expressed in this manner, the course of inactivation in each of twelve similar experiments was substantially linear, but, as the numbers of lesions produced is not a simple function of the virus concentration, we regard this as fortuitous. It is clear that the freshly prepared virus from frozen sap was less affected than the older preparation; this difference was regularly observed, although it is not always as great as in the experiment set out in Fig. 1.

The rate at which infectivity is lost in the presence of citrate depends greatly on the temperature; an increase of 3° in the range 15–22° approximately halved the number of lesions produced when conditions were used which decreased the number of lesions given by samples exposed at 15° to about one-quarter of that given by an unexposed control. In the pH range 6–7·5, the virus lost

little infectivity over short periods either in the absence of buffer or in 0·02 M glucosamine buffer, but small changes in pH affected inactivation by citrate. Table 4 records two experiments, showing that the rate of inactivation depends most closely on pH at about 6, the value at which inactivation by 0·1 M phosphate is also most influenced by pH changes.

Table 4. *Effect of* pH *changes on inactivation by* 0·02 M-*citrate*

(Mixtures of virus at 1·4 g./l. and citrate at the specified pH were kept for 24 hr. at 21° before dilution and testing for infectivity.)

Exp. 1			Exp. 2		
	Mean lesions per half-leaf at			Mean lesions per half-leaf at	
pH	5 mg./l.	0·5 mg./l.	pH	5 mg./l.	0·5 mg./l.
5·5	108	38	5·6	77	27
6·0	52	14	5·8	60	13
6·5	12	1	6·0	48	14
7·0	7·5	0·5	6·2	35	8·5
5·5*	132	41	5·6*	80	22
7·0*	109	34	6·2*	80	25

* Citrate added immediately before diluting for infectivity tests.

In every experiment in which the concentration of citrate was varied between 0·05 and 0·003 M, a concentration was found which had maximum inactivating effect, and solutions both stronger and weaker inactivated less. This optimum concentration was not constant and varied, not only from virus preparation to preparation, but also with one preparation when tested again after an interval. Maximum inactivation usually occurred with a citrate concentration about 0·02 M, and, as the two experiments in Table 5 show, the

Table 5. *Effect of concentration of citrate on extent of inactivation*

(Mixtures of virus at 1·4 g./l. and citrate or water were kept for 24 hr. at 21° before dilution and testing for infectivity.)

Exp. 1			Exp. 2		
	Mean lesions per half-leaf at			Mean lesions per half-leaf at	
Molarity of citrate	2 mg./l.	0·2 mg./l.	Molarity of citrate	2 mg./l.	0·2 mg./l.
0·04	42	5	0·05	100	48
0·02	29	3	0·02	3	0·2
0·01	8	2	0·008	27	8
0·005	37	8	0·0032	175	74
0·0025	91	13	0·0	177	100
0·0	74	12			

maximum can be well defined. The obvious interpretation of such a maximum is that the virus better resists the inactivating effect of citrate when the concentration of ions in the environment is increased. We therefore made many experiments in which the ionic concentration was increased by other salts.

These have given very variable results, but Exp. 1 in Table 6 is typical of about one-half of those made. From this it is clear that both 0·02 M sodium chloride and 0·02 M calcium chloride caused little inactivation themselves and protected the virus from inactivation by citrate. That they do not invariably

Table 6. *Effect of* NaCl *and* $CaCl_2$ *on inactivation by citrate*

(Virus at 0·6 g./l. was incubated for 48 hr. at pH 5·8 and 15°.)

Exp. 1			Exp. 2		
	Mean lesions per half-leaf at			Mean lesions per half-leaf at	
Solution used	2 mg./l.	0·2 mg./l.	Solution used	2 mg./l.	0·2 mg./l.
0·02 M citrate	24	2	0·08 M citrate	87	23
0·02 M-NaCl	77	16	0·02 M citrate	8	1
0·02 M-$CaCl_2$	74	13	0·02 M citrate + 0·05 M-NaCl	63	9
Citrate + NaCl	66	9	0·008 M citrate	36	9
Citrate + $CaCl_2$	75	16	0·008 M citrate + 0·05 M-NaCl	2	0
Water	103	20	Water	113	24

do so is partly explained by the results shown in Exp. 2 in Table 6, from which it seems that whereas 0·05 M sodium chloride protected the virus from the inactivating effect of 0·02 M citrate, it enhanced the effect of 0·008 M citrate. Table 6 again shows an optimum concentration for inactivation. The inconstancy of the citrate concentration needed for maximum inactivation has already been mentioned; presumably this value affects the concentration of sodium chloride needed to inhibit or enhance the inactivating effect of citrate, and the interaction of these two factors, each of which has variable effects depending on concentration, seems sufficient to explain our often irregular results.

It is not only salts that seem to affect inactivation by citrate. Of the two antiseptics tried, the rate of inactivation by 0·02 M citrate at pH 5·8 and 18° was slightly increased by the presence of saturated chloroform and was considerably retarded by saturation with thymol. Traces of chloroform were present in many of the experiments reported here, but thymol was not used as a disinfectant in any preparation destined for experiments with citrate.

A further variable that affects inactivation by citrate is the concentration of virus, and within the range we studied, inactivation was favoured by increased concentration. One experiment is shown in Table 7; virus at

Table 7. *Effect of initial virus concentration on inactivation by citrate*

(The fluids were kept for 48 hr. at pH 5·9 and 16°.)

Initial virus concentration (g./l.)	Treatment	Mean lesions per half-leaf at 2 mg./l.	Mean lesions per half-leaf at 0·2 mg./l.
0·66	Water	30	6·5
	0·02 M citrate	12	2
	0·02 M citrate added before testing	40	5
0·066	Water	39	5·5
	0·02 M citrate	32	6
	0·02 M citrate added before testing	69	10

0·66 g./l. incubated for 48 hr. at 16° produced only one-third of the number of lesions produced by the water control, whereas virus at 0·066 g./l. still produced almost as many lesions as did the water control. Table 7 also illustrates a phenomenon that we have frequently, though not invariably encountered; namely, that preparations exposed momentarily to citrate and the diluted, or mixed with diluted citrate, produced more lesions than corresponding water controls. The phenomenon complicates the interpretations of lesion counts so that comparisons with water controls may suggest less loss of infectivity than has actually occurred. The results from experiments in which the initial concentration of virus exposed to citrate was varied suggest that the rate of inactivation diminishes proportionally to the concentration of virus, which, at first sight, seems incompatible with the approximately linear relationship between time and percentage infectivity, which is illustrated in Fig. 1. However, there is no necessary incompatibility, for in none of the experiments is the relationship between lesion numbers and amounts of infective virus known; also, we do not know how the presence of variable quantities of non-infective particles affects the stability of infective particles towards citrate or influences the numbers of lesions produced by a given quantity of infective virus. The greater stability in dilute solutions is unusual with proteins, and is not found when this tobacco necrosis virus is exposed to agents other than citrate. The inactivation caused by neutral phosphate, for example, proceeds more rapidly in dilute than in concentrated virus solutions (Bawden & Pirie, 1945*a*). To quote one experiment: when samples of the same preparation at concentrations of 2·0, 0·5 and 0·11 g./l. were exposed to 0·1 M phosphate at pH 6·6 and 18° for 7 hr., they gave respectively an average of 20, 3 and 1 lesions per half-leaf at a dilution of 10 g./l., whereas a control to which phosphate was added immediately before dilution gave 62.

DISCUSSION

The results of our experiments on factors affecting the infectivity of the Rothamsted tobacco necrosis virus show how closely the effects of any given treatment depend on the immediate environment of the virus when the treatment is applied. In this paper we show that freezing infected leaves before they are minced to extract the virus leads to a product with much lower infectivity than if leaves are minced fresh. This contrasts vividly with the results recorded in the preceding paper (Bawden & Pirie, 1950) on freezing infective sap; so far from decreasing infectivity, the initial freezing of the sap produces purified virus preparations with the highest infectivity per unit weight of dry matter that we have been able to prepare. No conclusive interpretation of these varied effects can be given, but various possibilities can be suggested. It may be that freezing in the leaves renders virus particles non-infective without affecting their serological activity, whereas something in expressed sap prevents this inactivation. This interpretation, however, does not fit well with the fact that the infectivity of extracts from frozen leaves is still further decreased when the leaves are washed with water before they are minced, for this suggests that some effect secondary to freezing is also in-

fluencing infectivity. More in keeping with this phenomenon is the concept that the virus occurs in the leaf as a mixture of infective and non-infective particles, whose relative extractibility differs in fluids of different compositions. Another possibility is that freezing the leaves may combine the virus with some inhibitor of infectivity with which it is juxtaposed in intact cells but from which it is separated by mincing and extraction of sap. If this be so, the inhibitor must be firmly combined with the virus and remain with it through all stages of purification. In the experiments described in the preceding paper (Bawden & Pirie, 1950) we show that this virus undergoes considerable increases in infectivity while lying in some kinds of plant sap, which suggests that some, at least, of the virus particles develop infectivity after the leaves have been macerated, perhaps because of contact with some activator or 'co-factor'. The interpretation that best fits all our results is that much of the virus in intact cells is non-infective and that freezing the leaves destroys, or causes to be lost from the leaves, materials that normally react with the virus to make it capable of initiating fresh infections.

The results of exposing purified preparations of this tobacco necrosis virus to citrate also show how greatly minor changes in the environment determine to what extent inactivation occurs. In this respect the Rothamsted tobacco necrosis virus is not unique, for the inactivation of potato virus *X* (Bawden & Crook, 1947) and of T_5 bacteriophage of *Escherichia coli* (Adams, 1949) by citrate and phosphate also depend on the presence of other substances. The inactivation of this tobacco necrosis virus by citrate and phosphate seems to differ from that of potato virus *X*, which loses not only infectivity but also all other characteristic properties when incubated with these ions. The serological activity of potato virus *X* is destroyed and it is denatured, the nucleic acid being split from the protein moiety. Preparations of the tobacco necrosis virus still retain their full serological activity after incubation with citrate or phosphate in conditions where all infectivity is destroyed. No precipitates of denatured protein separate from these preparations during the inactivation, and there is no evidence that nucleic acid is liberated; such non-infective preparations also still produce crystalline pellets when they are ultracentrifuged. Many agents are known that rob other viruses of their infectivity without affecting their physical or serological properties, and the action of citrate on the tobacco necrosis virus may be a further example of this phenomenon. However, with this tobacco necrosis virus, there is less evidence linking antigenicity and infectivity with one kind of particle than with other viruses that have been studied, and there is no proof that the serologically active crystallizable protein ever carries any infectivity. This material forms the bulk of purified preparations of the tobacco necrosis virus, and it may be that infective particles form only a minor part. If this be so, complete decomposition of the infective component would not necessarily lead to any detectable changes in the physical or serological properties of a preparation.

The experiments with citrate show that inactivation to various degrees can be expected in the conditions obtaining in plant sap. The sap from tobacco leaves, whether healthy or virus-infected, at the time when they are normally

used for a virus preparation, contains 20–30 g./l. of diffusible dry matter. The physiological state of the plant greatly influences both its amount and composition. No complete balance sheet of the components of this fluid has been made but a few figures can be given. Vickery & Abrahams (1949) found that the citrate content can range from 0·04 to 0·20 M according to the conditions of culture; they also found that there is usually more malic than citric acid in the leaf. We have found variable values for some other ions but 0·01, 0·02, 0·03 and 0·06 M may be given as representative for PO_4, Ca, Na and K respectively. These figures fall in or near the range we have found to influence infectivity *in vitro*, so that it is possible that variations in the physiological state of the plant affect the severity of symptoms and the amount of virus in the leaf because of inactivations similar to those described here. Many other factors besides those we have mentioned may influence the activity, for we know nothing about the effects of leaf sugars and simple nitrogen compounds or of components with larger molecular-weight.

REFERENCES

Adams, M. H. (1949). The stability of bacterial viruses in solutions of salts. *J. gen. Physiol.* **32**, 579.

Bawden, F. C. & Crook, E. M. (1947). Some properties of potato virus *X* in leaf extracts made in different ways. *Brit. J. exp. Path.* **28**, 403.

Bawden, F. C. & Pirie, N. W. (1945*a*). Further studies on the purification and properties of a virus causing tobacco necrosis. *Brit. J. exp. Path.* **26**, 277.

Bawden, F. C. & Pirie, N. W. (1945*b*). The separation and properties of tobacco mosaic virus in different states of aggregation. *Brit. J. exp. Path.* **26**, 294.

Bawden, F. C. & Pirie, N. W. (1950). Some factors affecting the activation of virus preparations made from tobacco leaves infected with a tobacco necrosis virus. *J. gen. Microbiol.* **4**, 464.

Bawden, F. C. & Roberts, F. M. (1947). The influence of light intensity on the susceptibility of plants to certain viruses. *Ann. appl. Biol.* **34**, 286.

Bawden, F. C. & Roberts, F. M. (1948). Photosynthesis and predisposition of plants to infection with certain viruses. *Ann. appl. Biol.* **35**, 418.

Masket, A. V. (1941). A quantity type rotor for the ultracentrifuge. *Rev. sci. Instrum.* **12**, 277.

Vickery, H. B. & Abrahams, M. D. (1949). The metabolism of the organic acids of tobacco leaves. II. Effect of culture of excised leaves in solutions of D-isocitrate and acetate. *J. biol. Chem.* **180**, 37.

(*Received* 25 *February* 1950)

A Preliminary Study of Ammonia Production by *Corynebacterium renale* and some other Pathogenic Bacteria

By R. LOVELL and D. G. HARVEY

Royal Veterinary College, London

SUMMARY: Urease is a constituent enzyme of *Corynebacterium renale* and appears to account for the bulk of its ammonia production. *C. renale* also contains an arginase and some amino-acid deaminases, but the former has not been fully characterized. Bovine urine supports the growth of a small inoculum of *C. renale* for a limited time but after growth has reached a maximum it diminishes rapidly and the ammonia and pH values increase. *C. renale* also contains uricase but its precise significance has not yet been determined.

Of the other bacteria studied, *C. ovis* has urease activity similar to *C. renale*, and *C. pyogenes* a stronger initial arginase. *C. equi* contains no appreciable urease or arginase, although it tends to form ammonia from glutamine.

Corynebacterium renale is the cause of a specific cystitis and pyelonephritis in cattle; the lesions in natural infections in cattle and in experimental infections in mice and rabbits after intravenous inoculation are localized in the medulla of the kidney (Lovell, 1946; Lovell & Cotchin, 1946; Feenstra, Thorp & Gray, 1949). This selective localization may, in part, be explained by the metabolic and enzyme activities of the organism, and a study of its ammonia-producing capacity was made because of the old observation that *C. renale* produces ammonia when grown in urine. A preliminary test was made to confirm this by growing a strain of *C. renale* in bovine urine and in peptone water containing 1 % (w/v) urea. After 48 hr. incubation at 37° flasks of uninoculated media and those in which the organism had been grown were filtered through Seitz filters and the filtrates examined for free ammonia, urea and creatine. The results are given in Table 1, and show that in bovine urine and in a urea medium *C. renale* breaks down the urea with the formation of free ammonia. The creatine content of the urine remained constant, but largely diminished in the urea medium probably because of the peptone present.

The ammonia-producing capacity of *C. renale* was therefore examined and a few comparative tests made with *C. ovis*, *C. equi*, *C. pyogenes* and *Bacterium coli*.

METHODS

The methods employed were:

(*a*) Washed suspensions of *C. renale* prepared from growth on agar slopes were seeded into sterile urine, peptone water containing 1 % urea, hydrolysed peptone water and peptone water. The urine was sterilized by Seitz-filtration, and the hydrolysed peptone was prepared from a stock solution which consisted of a mixture of 100 g. of Bactopeptone and 300 ml. of conc. hydrochloric acid (A.R.) which had been boiled under a reflux condenser for 48 hr. after which most of the acid had been removed by vacuum distillation; the solution was neutralized to pH 7 by NaOH, diluted to give approximately 1 % NaCl;

L-tryptophan was added to give a concentration equivalent to 1 g. for every 100 g. of peptone originally used. After inoculation all cultures were incubated at 37° and periodical examinations made. The number of living bacteria/ml. was determined by plate counts on nutrient agar and estimations made of the ammonia and urea contents.

Table 1. *Content of urea,* NH_3 *and creatine (in g./100 ml.) of filtrates of urine and urea medium before and after growth of* Corynebacterium renale *at* 37° *for* 48 *hr.*

	Bovine urine		Urea medium	
	Control	After growth	Control	After growth
Urea	2·82	0·12	0·72	Nil
Free ammonia	Nil	1·309	Nil	0·289
Creatine	0·186	0·186	0·21	0·0232

(*b*) A cell-free extract of *C. renale* was prepared by growing large quantities of *C. renale* on nutrient agar for 48 hr.; the growth was collected, washed with saline and 10 vol. of cold acetone added to 1 vol. of thick washed suspension; the mixture was stirred with a glass rod and filtered through paper and the filter-paper washed with more acetone. The deposit was scraped off the filter-paper, dried over calcium chloride and then ground to a fine powder. Samples of the powder were added to different nitrogenous substrates in a buffered solution consisting of a mixture of 6 ml. 0·1 M-$NaHCO_3$ and 12 ml. 0·1 M-KH_2PO_4, the volume of which was made up to 40 ml. with distilled water; when using tyrosine, cystine and uric acid the concentration of KH_2PO_4 and $NaHCO_3$ was doubled. In order to avoid confusion between possible D- and L-deaminases DL-amino-acids were used wherever possible. Each individual constituent was weighed to give a final concentration of 15 mM. When mixtures of the nitrogenous substrates were used 1 ml. quantities of each substrate was taken as required and 5 ml. of the total taken for estimation purposes.

The ammonia was estimated by either micro- or macro-methods with steam distillation (Kjeldahl) or aeration (Van Slyke). For the nitrogenous substrates and their mixtures the following procedure was adopted. Five ml. of the stock solution was mixed with 5 ml. of an aqueous suspension of the cell-free extract of *C. renale* made by adding 150 mg. of the powder to 60 ml. of distilled water; this was incubated 4 hr. at 37–40°; toluene was added as a preservative. After incubation, the solution was treated with 5 ml. of saturated K_2CO_3 solution in a modified micro-Kjeldahl apparatus and the ammonia distilled into 5 ml. of 2 % boric acid + bromocresol blue and the final solution titrated with 0·01 N-H_2SO_4. The urea titrations were made by the Van Slyke urease method; the creatine and creatinine by Folin's method as modified by Cole (1933) and his modification of Benedict's method was used for the titration of uric acid. The allantoin titration was that of Larson as described by Hawk, Oser & Summerson (1947).

RESULTS

Growth of Corynebacterium renale *in bovine urine and urea medium*

The results obtained by growth of *C. renale* in bovine urine are shown in Figs. 1 and 2. In the first experiment (Fig. 1) the initial inoculum was small, about 1500 viable bacteria/ml.; the number increased to $1{\cdot}5 \times 10^6$ at 48 hr. and then diminished till after 6 days' incubation only 100 organisms/ml. were viable. This coincided with a decrease in urea, from 1·25 to 0·25 g./100 ml. at the third day, whilst the ammonia content increased to a little over 0·5 g./100 ml. during the same period. The second experiment (Fig. 2) shows the results with a large inoculum. The initial viable count of 162,000 organisms/ml. fell almost in a straight line to zero; in this sample of urine the urea content was 2·65 g./ml. which fell to 2 g./ml. by the seventh day; there was a corresponding increase in ammonia.

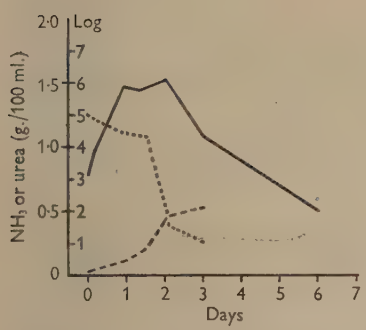


Fig. 1

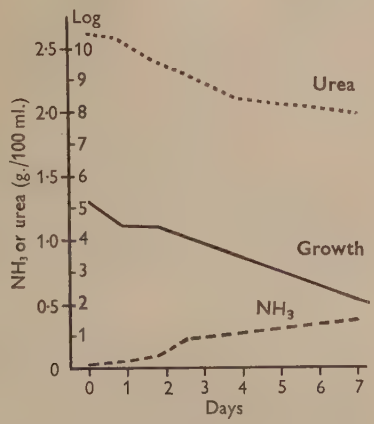


Fig. 2

Fig. 1. Urease activity and growth of *C. renale* in bovine urine. ——— Logarithmic count of growth; ····· urea in g./100 ml.; - - - - - - NH_3 in g./100 ml.

Fig. 2. Urease activity and growth of *C. renale* in bovine urine. ——— Logarithmic count of growth; ····· urea in g./100 ml.; - - - - - - NH_3 in g./100 ml.

Whether the initial inoculum of *C. renale* is large or small there is in bovine urine a steady though not always a rapid disappearance of the urea with a corresponding increase of ammonia concentration and pH value, the latter reaching 9·5.

In peptone water containing 1 % (w/v) urea similar results are obtained (Fig. 3). With an inoculum of 140,000 viable organisms/ml. there was a steady decrease in viable organisms, a fall in the urea content within 72 hr. and a corresponding increase in ammonia.

A different picture emerged when *C. renale* was grown in peptone water or in acid-hydrolysed peptone water (Fig. 4). In the former medium an inoculum approximating to that used in the urea medium grew and reached its peak at about 48 hr., thereafter falling gradually. Small amounts of ammonia were

present, from 13 to 30 mg./100 ml. In the acid-hydrolysed peptone water the viable organisms were rapidly killed and none were recovered after 48 hr. incubation. The ammonia content was irregular, varying from 6 to 23 mg./100 ml.

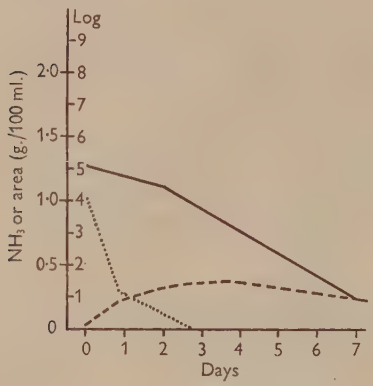


Fig. 3

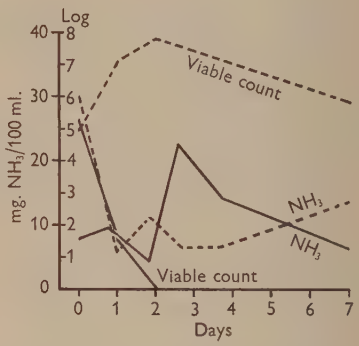


Fig. 4

Fig. 3. Urease activity and growth of *C. renale* in peptone water medium containing 1 % urea. ——— Logarithmic count of growth; ····· urea in g./100 ml.; - - - - - - NH_3 in g./100 ml.

Fig. 4. NH_3 production and growth of *C. renale* in peptone water and in acid-hydrolysed peptone water. - - - - - - Peptone water; ——— acid-hydrolysed peptone water.

Incubation of cell-free extract of Corynebacterium renale *in nitrogenous substrates*

The results obtained after incubation of the cell-free extract of *C. renale* in different nitrogenous substrates are shown in Table 2. They are compared with those obtained with similar products from *C. equi*, *C. ovis*, *C. pyogenes* and *Bact. coli*. Ammonia production by *C. renale*, although small, occurred with most of the substrates, the one exception being urea, which was rapidly and completely broken down. Prolonged incubation appeared to favour an arginase activity but beyond that ammonia production was slight. *C. ovis* also has urease, but *C. pyogenes* and *Bact. coli* have none; there is some evidence of an arginase of *C. pyogenes* but *C. equi* possesses neither urease nor arginase, it appears to break down (L+)-glutamine. Of the five organisms studied, *C. equi* and *Bact. coli* produced least ammonia when their cell-free extracts were incubated with a mixture of all the nitrogenous substances. This is difficult to reconcile with the results obtained when the strains were grown on the media described by Christensen (1946); in this medium, *C. renale*, *C. ovis* and *C. equi* gave evidence of urease activity, *C. pyogenes* gave a slight change in colour and *Bact. coli* failed to produce this change. When the phenol red was replaced by thymol blue (0·012 g./l.), positive reactions occurred with *C. renale* and *C. equi* and none with *C. ovis*, *C. pyogenes* and *Bact. coli*. In both media *C. renale* gave rapid and intense reactions.

Table 2. *The ammonia production (as μg. NH_3/mg. dry weight of cell-free enzyme preparation) after 4 hr. incubation with various substrates which were single or mixtures of nitrogenous compounds*

(a = results obtained with individual nitrogenous compounds; b, c and d = results obtained when different mixtures of compounds were used; . = no estimation made with this individual compound. All the amino-acids were DL except tyrosine, cystine, glutamine and arginine which were L. Cell-free extracts, when incubated in the buffer solution alone, gave slight traces of ammonia only.)

	C. renale				*C. equi*				*C. ovis*			*C. pyogenes*			*Bact. coli*		
Compound	*a*	*b*	*c*	*d*	*a*	*b*	*c*	*d*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
Glycine	5				2				.			.			.		
Alanine	5				3				.			.			.		
Valine	2	3			2	2			.			.			.	1	
Leucine	6				2				.			.			.		
Proline	4				2				.			.			.		
Hydroxyproline	4				1				.			.			.		
Isoleucine	4				2				.			.			.		
Norleucine	3	1			2	5			.			.			.	0	
Phenylalanine	3				3				.			.			.		
Tyrosine	3				3				.			.			.		
Lysine	2				3				.			.			.		
Histidine	0	6	10		2	4	4		.	9		.	8		.	6	
Ornithine	2				9				.			.			.		
Tryptophan	6				3				.			.			.		
Serine	4			8	3			1	.		9	.		10	.		3
Threonine	3	3			2	1			.			.			.	2	
Methionine	2				2				.			.			.		
Cystine	2				1				.			.			.		
Aspartic acid	3				0				.			.			.		
Glutamic acid	3	4			3	9			.			.			.	7	
Asparagine	3				5				.			.			.		
Glutamine	4				19				.			.			.		
Arginine	6				3				5			31			.		
Urea	84				7				83			4			.		
Creatinine	5				4				.			.			.		
Creatine	2	25			2	8			.	41		.	4		.		
Uric acid	3				<1				.			.			.		
Allantoin	2				<1				.			.			.		
Guanidine	2				32				.			.			.		

The arginase activity was studied by incubating 25 mg. of cell-free extract in a solution containing 50 ml. of a solution of 0·05 M arginine, 5 ml. of 0·6 % KH_2PO_4 and 0·45 ml. of 0·01 M-$MnSO_4$, and water to 100 ml.; toluene was added as preservative. A control flask omitting the $MnSO_4$ solution was included and the mixtures were incubated at 37° for 7 days. With the *C. renale* extract (Fig. 5) there was a steady increase in ammonia production, enhanced by the $MnSO_4$. Comparable extracts from *C. equi* and *Bact. coli* produced little ammonia, with no significant increase by $MnSO_4$. The arginase activity of *C. renale* was further demonstrated by the recovery of ornithuric acid after 3 days' incubation. This was effected by boiling the opalescent solution with 2–3 g. of kieselguhr for 30 sec., filtering and concentrating *in vacuo* to 25–30 ml.; the concentrate was treated with 1 ml. benzoyl chloride and excess NaOH, acidified with 5 % sulphuric acid, shaken with 5 × 20 ml. of ether and allowed

to cool. Impure ornithuric acid (dibenzoylornithine) was filtered off and recrystallized from dilute aqueous ethanol in colourless needles, M.P. 187–188° (uncorr.). Hills (1940) gave the melting-point as 182° and quoted references giving a range of 182–189°. The yield from 1·052 g. of (L+)-arginine was 500 mg. (55 % of theoretical). The once-recrystallized sample contained 7·2 % N; $C_{19}H_{20}O_4N_2$ requires 8·2 % N. Yellow-orange crystalline precipitates

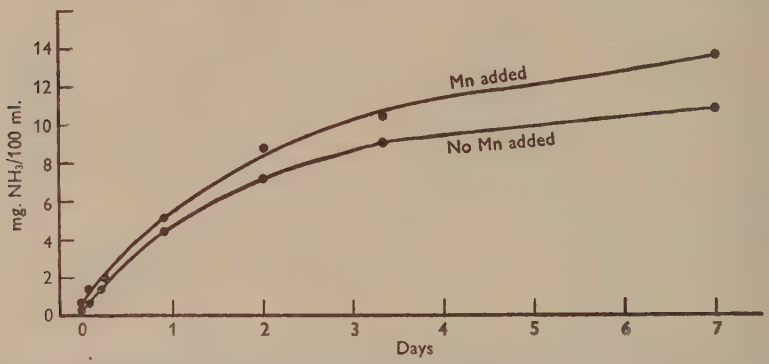


Fig. 5. Decomposition of arginine and production of NH_3 by cell-free extract of *C. renale* with and without added manganese.

were obtained from the *C. equi* and *Bact. coli* flasks when they were clarified and treated with 5 % flavianic acid. These precipitates decomposed at 260–270° with no sharp melting-points. The nitrogen analysis figures indicate that these substances were probably mixtures; *Bact. coli* has already been shown to possess an arginine decarboxylase activity giving rise to agmatine (Gale, 1940*a*, *b*).

The breakdown of nitrogenous constituents in human urine by cell-free extracts

A sample of human urine at pH 7 was divided into portions of 50 ml. and to three of them were added 25 mg. of the cell-free extracts of *C. renale*, *C. equi* and *Bact. coli* respectively. After 40 hr. incubation at 37° the main nitrogenous constituents were estimated in all four samples (Table 3).

Table 3. *Nitrogenous constituents of human urine after incubation with cell-free extracts of* Corynebacterium renale, C. equi *and* Bacterium coli

	Actual values of control sample (mg./100 ml.)	Percentage recovery when incubated with cell-free extracts of		
		C. renale	*C. equi*	*Bact. coli*
NH_3	42·5	1700	80	40
Urea	1165	5	100	—
Creatinine	97·5	99–100	99–100	99–100
Creatine	23·7	99–100	99–100	99–100
Uric acid	10·9	50	99–100	99
Allantoin	3·1	155	99–100	100
pH	7·0	8·8	7·1	7·1

The cell-free extract of *C. renale* attacked urea with a corresponding increase in the ammonia content. There was also a fall in uric acid with an increase in allantoin. Only slight changes occurred with extracts of *C. equi* and *Bact. coli*.

DISCUSSION

The ability of *C. renale* to form ammonia from various nitrogenous substrates undoubtedly lies in its urease activity. In common with the other bacteria studied it forms ammonia from a series of amino-acids, but in this respect its activity is slight. Proteolytic activity is also small and any ammonia produced by this means probably comes from the hydrolysis of amide nitrogen or by a primary proteolytic breakdown of the molecule followed by deaminase activity. This may be compared with the protease isolated from a streptococcus by Stevens & West (1922) and with the action of *C. equi* on glutamine.

We have not yet determined whether the arginase possessed by *C. renale* is a monohydrolase or dihydrolase. One practical difficulty is the wide pH range of the urease present, which we tentatively suggest is 5–9. If the arginase activity is optimal at pH $9 \pm 0{\cdot}5$ (Folley & Greenbaum (1948) suggest it is 9·45), then it may be possible to isolate an intermediate product such as urea which may be formed if the arginase is a normal monohydrolase type (Hills, 1940). There is no evidence that *C. renale* forms urea as an intermediate from compounds containing the $=N-\overset{|}{C}-N=$ linkage, such as creatine, creatinine, uric acid and allantoin. It is therefore unlike *C. ureafaciens* (Krebs & Eggleston, 1939). The only other enzyme activity concerned with urinary nitrogenous products, other than amino-acids, is uricase, an enzyme whose activity would be enhanced by the presence of the urea/urease system. Uricase is present in *C. renale* in sufficient amount to cause the disappearance of 50 % of uric acid in human urine at pH 7 after 40 hr. incubation. Further study of this isolated fact might reveal a linkage between the tissue of the host and the metabolic activities of the particular pathogen (Fildes, 1934–5; Knight, 1936). The activity of *C. renale* may be bound up with enzyme systems other than those which produce ammonia. Gale (1940*a*, *b*) showed that *Bact. coli* possesses a decarboxylase capable of converting arginine into agmatine, and although there is no evidence of agmatine formation by *C. renale*, other natural bases may be revealed which are harmful to the kidney tissue.

The differences in the growth curves in bovine urine from small and large inocula may be due to the rapid increase in ammonia production and pH value, produced by the larger concentration of urease. It is useless to speculate with a medium such as urine, as each sample is different, and it may be that any essential metabolites present are inadequate for small inocula and the growth thereby influenced in that way.

It is hoped to continue the study of the enzyme characteristics of *C. renale*, and one plan consists of varying the constituent nutrients. Hills (1940) noted that the relative concentrations of arginase and urease were sometimes reversed when growing staphylococci on special media. The main object is a study of the factors and conditions responsible for the localization and

multiplication of *C. renale* in the kidney medulla; it may lead to a fuller understanding of its pathogenesis and of the general problem of tissue localization.

We are indebted to Prof. C. Rimington and Dr G. Brownlee for gifts of some amino-acids and derivatives and to Messrs E. Langford and V. Drummond for valuable technical assistance.

REFERENCES

Christensen, W. B. (1946). Urea decomposition as a means of differentiating *Proteus* and *Paracolon* cultures from each other and from *Salmonella* and *Shigella* types. *J. Bact.* **52**, 461.

Cole, S. W. (1933). *Practical physiological chemistry*, 9th ed. Cambridge: Heffer and Sons.

Feenstra, E. S., Thorp Jr., F. & Gray, M. L. (1949). Pathogenicity of *Corynebacterium renale* for rabbits. *Amer. J. vet. Res.* **10**, 12.

Fildes, P. (1934–5). Some medical and other aspects of bacterial chemistry. *Proc. R. Soc. Med.* **28**, 79.

Folley, S. J. & Greenbaum, A. L. (1948). Determination of the arginase activities of homogenates of liver and mammary gland; effects of pH and substrate concentration and especially of activation by divalent metal ions. *Biochem. J.* **43**, 537.

Gale, E. F. (1940 *a*). The production of amines by bacteria. I. The decarboxylation of amino-acids by strains of *Bacterium coli*. *Biochem. J.* **34**, 392.

Gale, E. F. (1940 *b*). The production of amines by bacteria. III. The production of putrescine from L-(+)-arginine by *Bacterium coli* in symbiosis with *Streptococcus faecalis*. *Biochem. J.* **34**, 853.

Hawk, P. B., Oser, B. L. & Summerson, W. H. (1947). *Practical physiological chemistry*, 12th ed. York, Pa., U.S.A.: Mapel Press Company.

Hills, G. M. (1940). Ammonia production by pathogenic bacteria. *Biochem. J.* **34**, 1057.

Knight, B. C. G. J. (1936). Bacterial nutrition. *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 210.

Krebs, H. A. & Eggleston, L. V. (1939). Bacterial urea formation (metabolism of *Corynebacterium ureafaciens*). *Enzymologia*, **7**, 310.

Lovell, R. (1946). Studies on *Corynebacterium renale*. I. A systematic study of a number of strains. *J. comp. Path.* **56**, 196.

Lovell, R. & Cotchin, E. (1946). Studies on *Corynebacterium renale*. II. The experimental pathogenicity for mice. *J. comp. Path.* **56**, 205.

Stevens, F. A. & West, R. (1922). The peptase, lipase and invertase of hemolytic *Streptococcus*. *J. exp. Med.* **35**, 823.

(*Received* 3 *March* 1950)

Identification of Characteristic Extracellular Ninhydrin-Positive Substances Produced by some Bacteria

By A. J. WOIWOD and H. PROOM

The Wellcome Research Laboratories, Beckenham, Kent

SUMMARY: When certain species of bacteria are grown in an acid-hydrolysate of casein medium, ninhydrin-positive substances which were not present in the uninoculated medium appear on chromatograms of the culture filtrates. *Shigella paradysenteriae* and *Escherichia coli* produce γ-aminobutyric acid by decarboxylation of glutamic acid. The substance produced by *Serratia marcesens* resists acid hydrolysis, gives a positive Sakaguchi reaction and matches arginine on two-dimensional chromatography. The substances produced by *Clostridium sporogenes*, *Cl. bifermentans* and *Cl. sordellii*, but not by any of the other species of Clostridia examined, are δ-aminopentanoic acid, probably derived from proline, and α- and γ-aminobutyric acids produced by unknown mechanisms. *Proteus vulgaris* and *Clostridium tetani* each produce two polypeptides containing a high proportion of amino-acids in the groups valine/methionine and leucine/*iso*leucine. These two pairs of polypeptides are similar in R_F value and gross amino-acid composition. *Staphylococcus aureus* produces α-aminobutyric acid which may be derived from threonine.

By partition paper chromatography we showed previously (Proom & Woiwod, 1949) that certain species of bacteria, grown in an acid-hydrolysed casein medium, produced extracellular ninhydrin-positive substances. The production of a given substance was often associated with a particular group or species of bacteria and its appearance was concurrent with other changes on the chromatogram.

The substances produced by *Serratia marcesens*, *Shigella paradysenteriae*, *Proteus vulgaris* and *Clostridium tetani* were further examined and identified as arginine, γ-aminobutyric acid and polypeptides respectively (Woiwod & Proom, 1949). The present paper reports more fully the identification and characterization of these substances together with those produced by *Escherichia coli*, *Clostridium bifermentans* and *Staphylococcus aureus*. The possible value of these observations in bacterial classification will be discussed in a subsequent paper.

METHODS

Cultures in acid-hydrolysed casein medium were grown at 37° as previously described (Proom & Woiwod, 1949) and the culture filtrates were examined by the chromatographic technique of Woiwod (1949*a*). Single-dimensional chromatograms were run on sheets of Whatman No. 4 paper (*c.* 22 in. × 18 in.) with *n*-butanol + acetic acid as the solvent.

Usually four sheets were run with each culture filtrate, each sheet having thirty-five channels, with 0·015 ml. of filtrate for each channel put on the paper by means of a micrometer syringe. After running and drying the chromatograms the ninhydrin-positive material to be investigated was located by means of its fluorescence under ultra-violet light. Strips of filter-paper with

the material so located were cut from the papers and soaked overnight in water. The solutions thus obtained were pooled and the pool divided into two equal portions. Each portion was placed in a 10 ml. round-bottom flask having a ground-glass neck, and evaporated to dryness on a water-bath under reduced pressure. One portion was then hydrolysed by refluxing at atmospheric pressure with about 5 ml. of *c.* 6 N-HCl for 70 hr. using an air condenser. The acid was then removed by drying-down repeatedly with distilled water *in vacuo* on a water-bath. The residue was dissolved in a small volume of water. The dried but unhydrolysed portion of material was redissolved in the same volume. A strip of blank paper of approximately the same size was also extracted with water and the extract evaporated to dryness and hydrolysed. This was to control effects due to the possible presence of polypeptide impurities in the paper (Wynn, 1949). The solutions were then further investigated.

RESULTS

The polypeptides produced by Proteus vulgaris *and* Clostridium tetani

In the culture filtrates from all the strains of *Cl. tetani* and *Pr. vulgaris* examined, ninhydrin-positive material was observed which moved faster than leucine on the single-dimensional chromatogram with *n*-butanol + acetic acid as solvent. Two such spots were observed with both organisms, the slower-moving spot giving a considerably weaker colour reaction with ninhydrin than did the faster-moving spot. It was sometimes necessary to load the chromatogram heavily with culture-filtrate in order to demonstrate the presence of this slower-moving material. This weaker reaction with ninhydrin does not, of course, necessarily reflect the actual concentration of material on the paper.

Culture-filtrates were chromatographed as previously described, and strips of paper containing the individual spots were cut and eluted with water. The hydrolysed and unhydrolysed materials were run on single-dimensional chromatograms; the results are shown in Pl. 1, fig. 1. It is evident that on hydrolysis the materials of both the faster- and the slower-moving spots gave a number of amino-acids. The exact amino-acid composition of each hydrolysate was not ascertained, but the acids present were mainly in the leucine and valine groups (leucine, *iso*leucine, phenylalanine, methionine and valine). There was also a similarity in the gross amino-acid composition of all four hydrolysates. It can also be seen from Pl. 1, fig. 1 that the slower-moving spot from *Pr. vulgaris* was no longer visible on the chromatogram after elution from the paper. After hydrolysis of this spot, however, besides the amino-acids arising from hydrolysis of the original polypeptide, unchanged polypeptide was also again visible. This effect was observed on a number of occasions, but the reason for this behaviour is not known. It may perhaps be that the substances are rendered insoluble during the evaporation procedure after elution from the paper. Alternatively, some chemical change may occur when the chromatogram is dried before elution, which does not necessarily affect the solubility but only the reaction of the eluted material with ninhydrin. This latter mechanism would explain the difficulty of demonstrating the slower-moving spot on two-dimensional chromatograms.

In view of the similarity in R_F values and gross amino-acid composition of the corresponding pairs of polypeptides produced by both organisms, attempts were made by means of two-dimensional chromatography to establish their identity. Culture filtrates from *Pr. vulgaris* and *Cl. tetani* were freeze-dried and concentrated by redissolving in one-tenth of their original volume of distilled water. They were examined by two-dimensional chromatography on No. 4 Whatman paper using *n*-butanol + acetic acid followed by *m*-cresol and also benzyl alcohol. The faster-moving spot from both organisms occupied the same position on all the chromatograms. This fact, in conjunction with the similarity in the gross amino-acid composition of their hydrolysates, supports the view that the faster-moving polypeptides produced by *Pr. vulgaris* and *Cl. tetani* are chemically identical. The slower-moving spots, however, could not be detected on the two-dimensional chromatograms, and it was not possible further to load the chromatograms without smearing due to increased salt content. Culture-filtrates were therefore de-salted by the method of Consden, Gordon & Martin (1947). They were then concentrated by freeze-drying and redissolved in a small volume of water. It was still, however, not possible to demonstrate the presence of the slower-moving material on the two-dimensional chromatogram, although it was visible at the end of the initial *n*-butanol + acetic acid run. This supported the view that heating the chromatogram caused a change either in ninhydrin reactivity or solubility, at least in the case of the slower-moving material.

The production of γ-aminobutyric acid by Shigella paradysenteriae *and* Escherichia coli

Chromatograms of filtrates from cultures of *Sh. paradysenteriae* and *Esch. coli*, after being kept for 7 days at pH 4·5, showed a strong ninhydrin-positive spot in approximately the same position as tyrosine on the *n*-butanol + acetic acid chromatogram. The intensity of this spot was inversely related to the intensity of the glutamic acid spot (Proom & Woiwod, 1949). Culture filtrates were chromatographed as previously described, and strips of paper containing the unidentified material were cut out and eluted with water. The hydrolysed and unhydrolysed materials were further investigated. In searching for possible amino-acids to correspond in position with the unhydrolysed material, it was observed that its position on single-dimensional chromatograms could be closely matched with γ-aminobutyric acid. Two-dimensional chromatography with *n*-butanol + acetic acid followed by *m*-cresol separated the unhydrolysed material into a ninhydrin-positive substance and a fluorescent ninhydrin-negative substance which apparently had the same R_F values in *n*-butanol + acetic acid, although the latter material preceded the ninhydrin-positive material in *m*-cresol. Two samples of synthetic γ-aminobutyric acid from different sources also showed the presence of the fluorescent ninhydrin-negative material (Pl. 1, fig. 2). This suggests that it is not an impurity. A possible mechanism for its formation is ring-closure with the formation of a lactam. Whether the material is, in fact, the lactam of γ-aminobutyric acid is being investigated. The material produced by the organism, after hydrolysis and

two-dimensional chromatography (*n*-butanol + acetic acid followed by *m*-cresol) showed these two spots, together with two further fluorescent spots, only one of which was ninhydrin-positive. A similar picture was obtained when synthetic γ-aminobutyric acid was subjected to the same acid treatment. The chemistry of the production of these two spots has not yet been elucidated. However, it is clear that the material from *Sh. paradysenteriae* and synthetic γ-aminobutyric acid are identical. Confirmatory evidence was obtained by the procedure of Crumpler & Dent (1949), in which the paper chromatogram is dusted with basic copper carbonate along the path the amino-acids will travel during chromatography. Those which form copper salts, and this includes all α-amino-acids (Woiwod, 1949*b*), will run to one side and fail to react with ninhydrin, whereas non α-amino-acids appear in their normal positions on the chromatogram. It was found simpler to treat the paper by spraying with a 0·05 % (w/v) solution of cupric acetate (A.R.) in ethanol instead of with dry copper carbonate, and allowing the paper to dry before putting on the samples for chromatography in the usual way. Between 40 and 50 ml. of this solution per sheet of Whatman No. 4 paper (*c.* 22 × 18 in.) gave the best results. All such copper-treated single-dimensional chromatograms were run with *m*-cresol as solvent, since no copper complexes are formed when *n*-butanol + acetic acid is used. The results obtained with *Sh. paradysenteriae* are shown in Pl. 2, fig. 3; it can be seen that γ-aminobutyric acid and the unknown material are the only substances to run on the chromatogram and their positions are identical. Similar results are obtained with *Esch. coli*. This evidence, together with that already given, can be taken as reasonable proof that the material produced by *Sh. paradysenteriae* and *Esch. coli* is γ-aminobutyric acid.

As the production of γ-aminobutyric acid occurs only at acid pH it seemed likely that it arose by decarboxylation of glutamic acid (Gale, 1940). This was shown to be the case, by the examination of filtrates from washed organisms of *Sh. paradysenteriae* and *Esch. coli* suspended in a 1 % solution of glutamic acid and incubated for 7 days at pH 4·5. In most cases the glutamic acid had completely disappeared or was much decreased, whilst γ-aminobutyric acid appeared.

The production of α- and γ-aminobutyric acids and δ-aminopentanoic acid by Clostridium bifermentans, Cl. sordellii *and* Cl. sporogenes

We have previously shown (Proom & Woiwod, 1949) that culture filtrates from all the strains of *Cl. bifermentans* and *Cl. sordellii* showed an increase in the strength of the valine/methionine spot and the appearance of a strong spot just before tyrosine on the single-dimensional chromatogram with *n*-butanol + acetic acid as solvent. We now find that this effect is also given by filtrates from *Cl. sporogenes* but not by filtrates of any of the other Clostridia examined, i.e. *Cl. perfringens*, *Cl. novyi*, *Cl. histolyticum*, *Cl. tetani*, *Cl. haemolyticum*, *Cl. septicum*, *Cl. capitovale*, *Cl. butyricum*, *Cl. sphenoides* and *Cl. tetanomorphum*.

The intensity of these spots from *Cl. bifermentans* and *Cl. sordellii* was inversely related to the intensity of the proline spot. In view of our experience with *Sh. paradysenteriae* it seemed worth while to consider possible mechanisms

by which ninhydrin-positive substances could be produced from proline. One such mechanism would be the opening of the proline ring and subsequent reduction to give δ-aminopentanoic acid as described by Stickland (1935) for *Cl. sporogenes*. The spot appearing in the valine position was identified as δ-aminopentanoic acid by comparing its position with an authentic sample on a single-dimensional chromatogram using *n*-butanol + acetic acid as solvent, and on a two-dimensional chromatogram developed by *n*-butanol + acetic acid followed by *m*-cresol (Pl. 2, fig. 4). Final proof was obtained by running two-dimensional chromatograms on paper treated with copper acetate before running with the second solvent (*m*-cresol). The suspected δ-aminopentanoic acid appeared in its usual position, being, like γ-aminobutyric acid, unable to form a copper complex. These experiments satisfactorily explained the relationship with proline of one of the ninhydrin-positive metabolic products seen on the chromatogram, but still left the strong spot near tyrosine unidentified. Two-dimensional chromatography of an eluate of this material revealed two substances present which from their positions appeared most likely to be α- and γ-aminobutyric acids. This was confirmed by running artificial mixtures of these substances in *n*-butanol + acetic acid followed by *m*-cresol with alanine, valine and proline as markers. An exact match was obtained. The γ-aminobutyric may have arisen from glutamic acid decarboxylase activity which would be relatively weak at the pH of the culture filtrate (*c.* pH 8·0). The mechanism of the α-aminobutyric acid formation is at present unknown.

The production of arginine by Serratia marcesens

When culture filtrates of *Ser. marcesens* were examined by paper chromatography a marked increase in the intensity of the ninhydrin reaction in the region of the basic amino-acids lysine, histidine and arginine was observed. Elution and chromatography of the material responsible for this failed to reveal more than one substance present, nor were any new spots observed after acid-hydrolysis. The material was not histidine or lysine, from which it could be separated by two-dimensional chromatography. From its position it appeared to be arginine and this was proved by two-dimensional chromatography using lysine and glycine as markers and a sample of synthetic arginine for comparison, and also by the fact that it gave a strong Sakaguchi reaction. The magnitude of the increase of arginine was not determined, and the mechanism of its formation is at present unknown.

The production of α-aminobutyric acid by Staphylococcus aureus

Culture filtrates of *Staph. aureus* showed a spot moving slightly slower than tyrosine on chromatography in *n*-butanol + acetic acid (Proom & Woiwod, 1949). This spot was identified as α-aminobutyric acid by two-dimensional chromatography of the eluate from single-dimensional chromatograms. This amino-acid has also been demonstrated inside the cells of this species by Dr B. A. Fry (personal communication) who washed the organisms, disrupted the cells in boiling-water and chromatographed the supernatant after centrifuging-off the cell debris. As with the anaerobic organisms, where we found

this amino-acid in the culture filtrates, it is not yet known how α-amino-butyric acid is formed. When washed suspensions of staphylococci were incubated with threonine and chromatographed, ninhydrin-positive material was observed which appeared to be α-aminobutyric acid. It is possible, therefore, that this acid is formed by removal of the hydroxyl group from threonine.

DISCUSSION

A number of the ninhydrin-positive materials seen on paper chromatograms of culture filtrates, and identified in the present work, have already been demonstrated by other techniques. The production of γ-aminobutyric acid by *Esch. coli* and *Sh. paradysenteriae* are examples of the specific decarboxylation of glutamic acid demonstrated by Gale (1940). Similarly, the production of δ-aminopentanoic acid by anaerobic organisms was demonstrated for *Cl. sporogenes* by Stickland (1935).

The production of polypeptides by *Cl. tetani* and *Pr. vulgaris* and of arginine by *Ser. marcesens* does not appear to have been observed previously. It is interesting to speculate whether the production of similar polypeptides by two such dissimilar organisms as *Cl. tetani* and *Pr. vulgaris* comes by decomposition of bacterial protein by similar proteolytic enzymes or whether these materials are first synthesized and then excreted by the organism.

REFERENCES

Consden, R., Gordon, A. H. & Martin, A. J. P. (1947). The identification of lower peptides in complex mixtures. *Biochem. J.* **41**, 590.

Crumpler, H. R. & Dent, C. E. (1949). A distinctive test for α-amino acids in paper chromatography. *Nature, Lond.*, **164**, 441.

Gale, E. F. (1940). The production of amines by bacteria. 1. The decarboxylation of amino-acids by strains of *Bacterium coli*. *Biochem. J.* **34**, 392.

Proom, H. & Woiwod, A. J. (1949). The examination, by partition paper chromatography, of the nitrogen metabolism of bacteria. *J. gen. Microbiol.* **3**, 319.

Stickland, L. H. (1935). Studies in the metabolism of the strict anaerobes. II. The reduction of proline. *Biochem. J.* **29**, 289.

Woiwod, A. J. (1949*a*). A technique for examining large numbers of bacterial cultural filtrates by partition chromatography. *J. gen. Microbiol.* **3**, 312.

Woiwod, A. J. (1949*b*). A method for the examination of micro amounts of amino nitrogen and its application to paper partition chromatography. *Biochem. J.* **45**, 412.

Woiwod, A. J. & Proom, H. (1949). Further observations on the group or species specific ninhydrin positive substances produced by bacteria. *J. gen. Microbiol.* **4**, Proc. v.

Wynn, V. (1949). A peptide-like contaminant of filter paper. *Nature, Lond.*, **164**, 445.

Fig. 1

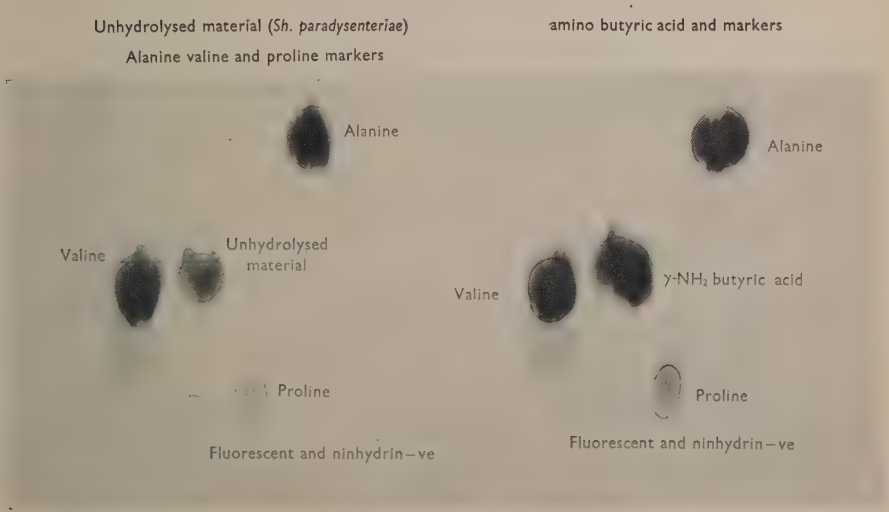


Fig. 2

A. J. Woiwod & H. Proom—Identification of characteristic extracellular ninhydrin-positive substances produced by some bacteria. Plate 1

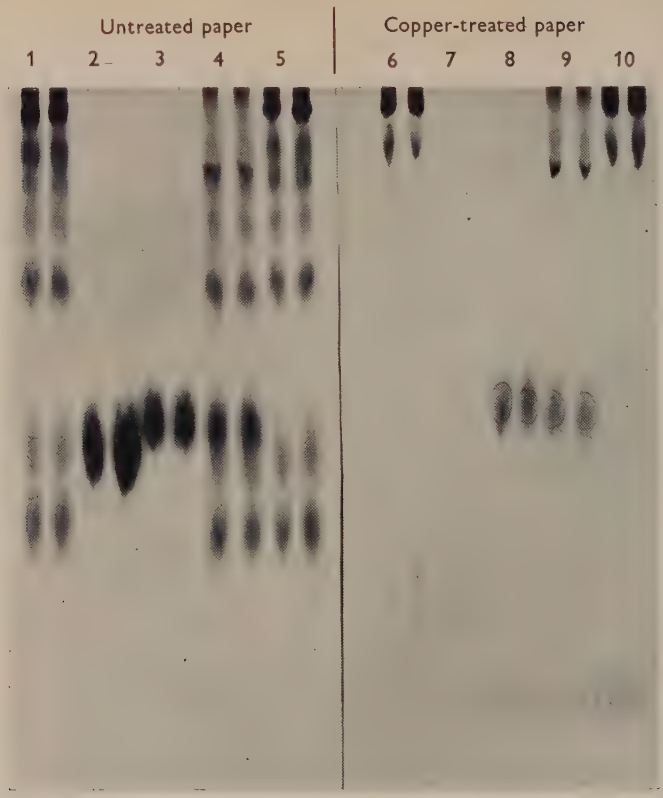


Fig. 3

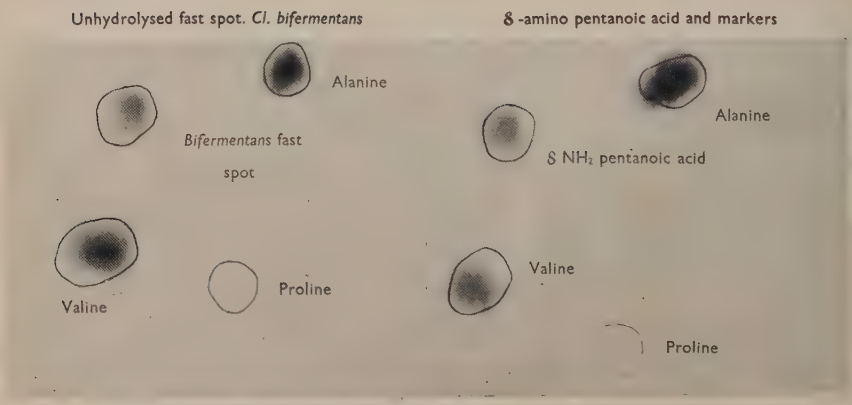


Fig. 4

A. J. Woiwod & H. Proom—Identification of characteristic extracellular ninhydrin-positive substances produced by some bacteria. Plate 2

EXPLANATION OF PLATES

Plate 1

Fig. 1. Single-dimensional chromatograms of peptides isolated from culture filtrates of *Pr. vulgaris* and *Cl. tetani*, before and after acid hydrolysis. 1, 5 and 9, *Pr. vulgaris* culture filtrate; 2, fast-moving peptide, unhydrolysed (*Pr. vulgaris*); 3, slow-moving peptide, unhydrolysed (*Pr. vulgaris*); 4, paper blank, unhydrolysed; 6, fast-moving peptide, hydrolysed (*Pr. vulgaris*); 7, slow-moving peptide, hydrolysed (*Pr. vulgaris*); 8, paper blank, hydrolysed; 13, 17, *Cl. tetani* culture filtrate; 10, slow-moving peptide, unhydrolysed (*Cl. tetani*); 11, fast-moving peptide, unhydrolysed (*Cl. tetani*); 12, paper blank, unhydrolysed; 14, slow-moving peptide, hydrolysed (*Cl. tetani*); 15, fast-moving peptide, hydrolysed (*Cl. tetani*); 16, paper blank, hydrolysed. A, lysine, histidine, arginine; B, aspartic acid; C, glycine, serine; D, glutamic acid, threonine; E, alanine; F, proline; G, tyrosine; H, methionine, valine; J, phenylalanine; K, leucine, *iso*leucine; L, slow-moving peptide; M, fast-moving peptide.

Fig. 2. Two-dimensional chromatograms of material isolated from a culture filtrate of *Sh. paradysenteriae* compared with synthetic γ-aminobutyric acid. Solvents: horizontal, right to left, *n*-butanol + acetic acid; vertical, *m*-cresol.

Plate 2

Fig. 3. Confirmation of γ-aminobutyric acid in culture filtrates of *Sh. paradysenteriae* by means of single-dimensional chromatograms run on filter-paper with and without copper treatment. 1, 5, 6 and 10, uninoculated medium; 2, 7, valine; 3, 8, synthetic γ-aminobutyric acid; 4, 9, *Sh. paradysenteriae* culture filtrate. Solvent, *m*-cresol.

Fig. 4. Two-dimensional chromatograms of material isolated from culture filtrate of *Cl. bifermentans* compared with a synthetic sample of δ-aminopentanoic acid. Solvents: horizontal, right to left, *n*-butanol + acetic acid; vertical, benzyl alcohol.

(*Received* 6 *March* 1950)

A Comparative Survey of the Nutrition and Physiology of Mesophilic Species in the Genus *Bacillus*

By B. C. J. G. KNIGHT and H. PROOM
The Wellcome Research Laboratories, Beckenham, Kent

SUMMARY: Two hundred and ninety-six strains of mesophilic species of the genus *Bacillus* were isolated from soil and examined for the characters described principally by Smith, Gordon & Clark (1946) as well as for some additional characters. Two hundred and forty-six belonged clearly to named species in the classification of Smith *et al.*, thirty-two strains were clearly intermediate between two species and eight strains remained unallocated. In addition, eleven other strains appeared to represent a previously undescribed species (Proom & Knight, 1950).

Ability to grow under strictly anaerobic conditions, to give the Gibson & Abdel-Malek (1945) test and to produce typical (lecithinase-like) or 'restricted' reactions with egg-yolk emulsion were valuable diagnostic characters in this genus.

A survey was made of the nutritional requirements of some 200 strains which included many of the newly isolated ones and representative laboratory strains. Typical nutritional patterns characterizing the species were found, with an unexpected degree of uniformity with the groups of strains examined; the number of nutritionally aberrant strains in each species was very small.

The characteristic nutritional patterns were:

B. subtilis, *B. licheniformis* and *B. megatherium* grew with ammonia as nitrogen source and in the absence of added growth factors.

B. cereus and *B. brevis* grew in absence of added growth factors but required mixtures of amino-acids instead of ammonia only.

B. pumilus and *B. polymyxa* both grew with ammonia + biotin, and *B. macerans* grew with ammonia + biotin + aneurin.

B. alvei required amino-acids + aneurin; *B. circulans* and *B. coagulans* required amino-acids and usually both aneurin and biotin; some strains of *B. circulans* had more complex requirements.

Some strains of *B. sphaericus* required amino-acids + aneurin, others required biotin as well, and all of the strains of *B. sphaericus* var. *fusiformis* required amino-acids + aneurin + biotin.

The strains of *B. pasteurii* were the most heterogeneous in their nutritional requirements, the components ammonium ion, amino-acids, aneurin, biotin and nicotinic acid being involved. All strains required amino-acids and aneurin; in addition, biotin or nicotinic acid and sometimes ammonium ion were required, depending on the particular strain.

There are few records of wide comparative surveys of bacterial genera which have made use not only of the classical bacteriological distinguishing tests and methods, but which have also included determinations of exact nutritional requirements, and of metabolic and serological characters. Intensive studies of particular species from these aspects have been somewhat more common; but even here comparative studies of numerous strains or clones of a species, when made, have only seldom included all the features noted above, so that well-rounded pictures of their biological properties are infrequent. The Culture Collection at these Laboratories, with the staff and services necessary for its maintenance and development, offered an opportunity to make comparative

surveys of large groups of organisms and to examine many aspects of their physiology.

It was hoped that such comparative surveys might yield information which would help to improve the diagnosis, differentiation and classification of bacteria as they came to hand. It was further hoped that from this material might come indications of natural relationship, thus helping to make classifications somewhat less arbitrary than they tend to be at present. A long-term objective is to accumulate information which might contribute to the study of taxonomic and evolutionary problems.

A more immediate objective has been to see to what extent nutritional requirements are characteristic of species as already differentiated by the classical bacteriological tests, many of which are empirical and obviously have different weights as definitive of species characters.

This is not the place to discuss the problem of species differentiation; some pertinent examples will appear in the sequel. Indeed it might be well at this point to state our standpoint in this respect. We believe that the question of species definition and differentiation among bacteria is best not argued as a metaphysical question, *a priori* as it were, but that the question of classification will be better worth discussion only when much more pertinent material is available. It is part of the purpose of the present work to supply some of this material. It is, of course, true that for practical purposes distinguishing characters, however empirically determined, are invaluable and that at least empirical classifications do have to be made for current use. But this does not preclude a desire to achieve better classifications, which will presumably be the better the more closely they reflect natural relationships.

In the present work two sources of organisms were used: (*a*) named cultures from collections and other laboratory strains; (*b*) strains freshly isolated from natural sources. In this way it was possible to see whether long-continued laboratory culture had modified any characters and to assess the homogeneity of groups of freshly isolated strains, which presumably represented the organisms occurring in nature.

The genus *Bacillus*, excluding thermophilic organisms, was chosen for our first survey for a number of reasons. It includes one organism frankly pathogenic to man and some animals, namely *B. anthracis*, one which is pathogenic for bees, *B. larvae*, a number of species of interest as producers of antibiotics (e.g. *B. subtilis*, *B. brevis*, *B. polymyxa*, *B. licheniformis*, *B. circulans*), and is a relatively well-defined genus. The investigations of Gibson (1934 *a*, *b*; 1935 *a*, *b*; 1944) and of Smith *et al.* (1946) provided a firm basis from which to start. The classification introduced by Smith *et al.* (1946) and used in Bergey's *Manual of Determinative Bacteriology* (6th edition, 1948) divides the genus into three groups on the basis of the morphology of the spores and sporangia, and into fifteen species and seven varieties on physiological grounds. This contrasts with the 157 differently named 'species' which were examined by Smith *et al.* and with the 26 pages of names and references of unallocated and insufficiently described strains which are recorded in the appendix to the genus in Bergey's *Manual*. We have found the Smith *et al.* (1946) classification workable and

useful, and have used it in identifying our newly isolated strains. These strains were then examined by tests not used by these workers, and in many cases the nutritional requirements of both laboratory and newly isolated strains were examined. On the basis of the results, the classification of the genus as it stands at present was then examined.

The exact nutrition of species of the genus *Bacillus* has, with few exceptions, been little studied; and no extensive comparative examination has been made. There are references in the earlier literature to the cultivation of one or a few strains of certain organisms, e.g. *B. subtilis*, on very simple media (e.g. ammonia or asparagine, glucose and salts), but when much of this work was done the role of very low concentrations of certain substances (essential metabolites) in promoting growth was not adequately recognized. It is not therefore always certain that the simple 'defined' media used were, in fact, free from organic growth factors, such as biotin, especially when the 'defined' media contained a natural product like asparagine, sucrose or glucose. More recently, since the recognition of the role in nutrition of various essential metabolites of the vitamin B group, this point has usually been recognized and adequately controlled in nutritional studies.

The nutrient requirements of certain strains of *B. anthracis* were studied by Gladstone (1939) and by Brewer, McCullough, Mills, Roessler, Herbst & Howe (1946). Since we have not studied this organism their observations may be summarized here. Gladstone used eight strains of *B. anthracis* obtained from the National Collection of Type Cultures; six grew well on a medium containing an array of amino-acids, salts and glucose; but no growth factors of the vitamin B group appeared to be needed. Gladstone (private communication, 1949) on repeating his earlier work with some six strains (including one or two of the earlier collection) found a requirement for aneurin and adenosine + adenylic acid. The adenosine and adenylic acid were important in initiating growth. Brewer *et al.* (1946) found that the strain 'Vollum, M-36' required aneurin in addition to a collection of amino-acids, salts, etc.; aneurin was the only growth factor required. Thus *B. anthracis* appears to require a collection of amino-acids and that at least some strains require aneurin, with adenosine and/or adenylic acid, the latter possibly as growth initiators or stimulators.

Other organisms of the genus *Bacillus* which have been examined for nutritional requirements and in which collections of strains were used include *B. alvei* and *B. para-alvei* (Katznelson & Lochhead, 1947), *B. macerans* and '*B. acetoaethylicus*' (Katznelson, 1944). Eight strains of *B. larvae* were examined by Lochhead (1942) and found to require aneurin and peptone; the peptone may have been a source of other nutrients besides amino-acids. The study of *B. larvae* was extended by Katznelson & Lochhead (1948), who found that the intact aneurin molecule was required by most strains, which also required various purines and pyrimidines; and some strains required strepogenin. We have not examined strains of *B. larvae* in the present work. Recently Cleverdon, Pelczar & Doetsch (1949*b*) examined the vitamin requirement of numerous strains of stenothermophilic (55–65°) organisms of the genus

Bacillus. With an acid-hydrolysed casein basal medium, the essential metabolites required as nutrients were aneurin, biotin and nicotinic acid. We have not examined organisms growing above 45°; the results of Cleverdon *et al.* (1949*b*) are considered below. Other references to nutritional requirements in this genus are cited below. In the present paper are recorded the results of an examination of the nutrition of some 200 newly isolated and laboratory strains.

EXPERIMENTAL

The isolation of strains of Bacillus *from soil*

Isolations from soil samples. Some hundred samples of soil from widely separated localities in the British Isles, and a few specimens from Europe, were examined. In order to make the study as representative as possible we isolated only one culture of any particular species from any one sample of soil; 296 strains were thus isolated. The pre-treatment of the soil and the use of differential media had a marked effect on the kinds of species which were isolated. The numbers and variety of species varied with the soil samples, but not as much as was expected.

To 10 ml. of medium contained in a $6 \times \frac{5}{8}$ in. test-tube sufficient soil was added to give a total volume of *c.* 15 ml. The tube was well shaken, and a loopful of suspension plated by streaking on an appropriate agar medium. When a tube of soil suspension was to be heated a thermometer was placed in the tube, or in a control tube containing the same volume of tap water, to ensure that the sample reached, and was kept at, the desired temperature for the appropriate time. Temperatures were controlled to $\pm 1°$.

After incubation of the inoculated plates single colonies were transferred to nutrient agar slopes; at the same time Gram-stained preparations were examined. After 2 days' incubation at 28 or 37° sporulating cultures were purified by plating. When a culture morphologically resembled the vegetative form of members of the genus it was left at room temperature, was examined at intervals for sporulation and discarded if this did not occur within 2 weeks. Of the new isolates obtained as described 10–20 % were asporogenous and were discarded. Special media alleged to induce sporulation made little difference to the number of asporogenous forms encountered and were not used routinely.

The species obtained by the differential isolation techniques

The results obtained by different methods of treating soil suspensions and plates may be summarized as follows:

(*a*) Direct plating of soil suspension in broth on nutrient agar followed by incubation at 37° for 2 days gave predominantly *B. subtilis* to the exclusion of other organisms. An occasional colony of *B. circulans*, *B. megatherium* or *B. cereus* was obtained.

(*b*) Soil suspension in broth plated on nutrient agar and incubated at 28° instead of 37° yielded *B. cereus* var. *mycoides* and *B. subtilis* as predominant organisms.

(*c*) Soil suspension in broth heated at 70° for 10 min. followed by plating on nutrient agar and incubation at 37° gave a mixture of *B. subtilis* and

B. circulans. Incubation at 28° instead of 37° gave a mixture of *B. cereus* var. *mycoides* and *B. subtilis*.

(*d*) Soil suspension in broth heated at 70° for 10 min., incubated in broth at 28° for 2 days, plated on nutrient agar and incubated at 37° for 2 days, gave a mixture of *B. cereus* and *B. megatherium* with occasional colonies of *B. subtilis*. Plating on nutrient agar and incubation at 28° instead of 37° gave a mixture of *B. cereus* and *B. megatherium* with some colonies of *B. cereus* var. *mycoides* and *B. subtilis*.

(*e*) Soil suspension in distilled water incubated at 37° for 3 days, followed by plating on nutrient agar and incubation at 37° produced a very mixed collection of colonies. *B. subtilis*, *B. cereus* and *B. megatherium*, although present, were fewer in number. *B. pumilus* and *B. circulans*, with occasional colonies of *B. brevis*, *B. macerans*, *B. alvei* (once) and *B. sphaericus*, were observed.

(*f*) Soil suspension in milk incubated for 3 days at 50° and plated on nutrient agar at 45° also gave a varied collection of organisms from which *B. coagulans* could be readily isolated.

(*g*) Soil suspension in 3·0 % glucose broth was heated at 65° for 10 min., incubated at 28° for 3 days, plated on nutrient agar and incubated at 28°. Selected colonies were grown in glucose broth and those cultures which produced acid and gas were plated on nutrient agar. This gave strains of *B. polymyxa*.

(*h*) Soil suspension in urea (10 or 2·0 %) broth was incubated for 2 days at 28° and plated on nutrient agar containing either 10 or 2·0 % urea. This treatment suppressed all organisms other than those of morphological group 3, and strains of *B. sphaericus* together with its varieties, *B. pasteurii* and occasional strains of *B. lentus*, were isolated.

After identification the new isolates were stored as freeze-dried desiccates.

Bacteriological methods used in examining the cultures

In order to compare our results with those of Smith *et al.* (1946) we used bacteriological media that resembled theirs as closely as practicable. Our nutrient broth containing *c.* 3 g. N/l. was prepared by the addition of papain digest of horse muscle containing *c.* 1·5 g. N/l. to a water extract of fresh horse muscle. Nutrient agar was prepared by adding 1·2 % agar (Davies Gelatin (N.Z.) Ltd.) to this nutrient broth.

Microscopical examination. Organisms grown on nutrient agar were Gram-stained and examined microscopically. The size of vegetative rods and of spores was not usually measured. Although typically large- and small-cell species exist it was observed that the effect of different media and strain variations made such measurements of little value in identification. There was, however, one notable exception. Smith *et al.* (1946) observed that *B. megatherium* and *B. cereus* could be distinguished from other members of morphological group 1 (see below) by the fact that the width in stained preparations of the vegetative rods of these two organisms was 0·9 μ. or greater; this we amply confirmed. The position and shape of the spore, the thickness of the spore-wall and the shape of the sporangium were found by Smith *et al.* to be of considerable value

in classification. We found little difficulty in allotting a new isolate to one or other of the three main morphological groups of the classification of Smith *et al.* These are: group 1, in which the sporangium is only slightly swollen, or not at all, by a thin-walled oval spore; group 2, in which the sporangium is swollen by a thick-walled oval spore; group 3, in which the sporangium is swollen by a thick-walled spherical spore.

Smith & Clark's (1937) observation that *B. megatherium* and *B. cereus*, in contradistinction to other species, when stained by Sudan III showed large fat globules, was confirmed. The later observation of Smith *et al.* (1946) that when stained by Hartman's (1940) method all members of the genus *Bacillus* showed stored fat, did not, in our experience, diminish the value of the original observation. As a routine we used the method described by Burdon, Stokes & Kimbrough (1942). With this technique *B. cereus* and *B. megatherium* showed many large fat globules, while other species had none or only a few small fat globules.

Macroscopical examination. The colonial appearance on nutrient agar and the nature of the growth on nutrient agar, glucose nitrate agar, glucose nutrient agar and potato, and in nutrient broth, were observed. The appearance of the growth and the colonial form were too variable to be of much use in the identification of species. The rapidity of dissociation on artificial media and the occurrence of numerous varieties, e.g. rough, smooth, mucoid, sporogenous, asporogenous, each with a distinct colonial appearance, is well known. It is illustrated by recent publications by Ledingham, Adams & Stanier (1945) and by Francis & Rippon (1949), who described such variants in *B. polymyxa*. It is, however, our impression that on first isolation from the soil each species has a predominant colonial form, suggesting that in the soil one variant is predominant. We were unable to confirm the observation of Smith *et al.* that the nature of the growth on glucose nitrate agar was of value in identification.

Physiological reactions. Strains were examined for the production of acid and gas from glucose, arabinose and xylose. In view of the observations of Smith *et al.* (1946) these sugars were, with a few exceptions, the only ones used in routine examination. Three types of sugar media were tried: medium *a*, peptone water; medium *b*, a liquid medium in which the sole source of nitrogen was ammonia; medium *c*, 1·2 % agar slopes made with medium *b*. These media were inoculated with a loopful of nutrient agar culture. The results obtained with medium *a* were irregular because of the ammonia produced from the peptone by many of the species. Medium *b* was inferior to *c* and often failed to support adequate growth. Medium *c* gave the most reproducible results. Many of the species tested required preformed amino-acids and specific growth factors as nutrients, and these substances were no doubt supplied by carry-over in the inoculum and as impurities in the agar.

Hydrolysis of casein, gelatin and starch was determined by the plate methods of Smith *et al.* (1946). The urea medium of Christensen (1946) containing phenol red indicator was used for the detection of urease.

The Voges-Proskauer (VP) reaction (acetylmethylcarbinol production) was determined by the method of Smith *et al.* (1946) after incubation at 28° for

2 and 6 days, except in the case of *B. coagulans* when incubation was carried out at 45°.

Reduction of nitrate to nitrite, utilization of citrate, growth in broth containing 4 % NaCl (NaCl broth) and growth on nutrient agar at pH 6·0 were all examined. All strains were tested for production of catalase, ability to grow anaerobically on nutrient agar in McIntosh-Fildes anaerobic jars, and for the production of gas from glucose under the semi-anaerobic conditions of the Gibson & Abdel-Malek (1945) test.

Tests for lecithinases or other enzymes visibly affecting egg-yolk emulsion were made on nutrient agar plates containing 10·0 % (v/v) of egg-yolk emulsion (Macfarlane, Oakley & Anderson, 1941). Two types of reactions were observed: (i) the reaction characteristic of such organisms as *Cl. perfringens* and *B. cereus* which give a zone of opalescence extending usually well beyond the area of growth, indicating easily diffusible enzymes. In these two organisms the effect is known to be due to C type lecithinases; (ii) a fainter reaction which was restricted to the medium immediately beneath the colony or streak of growth and was in general visible only when the growth was scraped away. This type of egg-yolk reaction was produced by *B. pumilus*, *B. polymyxa*, *B. macerans*, *B. alvei* and *B. brevis*, and will be referred to as a 'restricted' reaction. It may or may not be due to a lecithinase.

Nutritional requirements

General. The organisms were cultivated in *c.* 5 ml. lots of medium in 6 × 1 in. rimless boiling-tubes closed by loosely fitting aluminium caps. These tubes were incubated in a sloped position at about 10° from the horizontal, thus permitting relatively good aeration for these static cultures; the liquid medium was about 12 mm. thick at the deepest part and became progressively shallower towards the mouth of the tube.

The usual precautions to avoid contamination by traces of unwanted nutrients were adopted. All glassware was cleaned with chromic-sulphuric acid mixture, thoroughly washed with running tap water and finally with running distilled water. All chemicals were of highest commercial grade and further purified if necessary.

Inoculation and serial subcultivation. The starting-point for cultivation was a tube of freeze-dried cultures from the Collection. The culture was seeded into nutrient broth and from that to agar slopes. Since ten to twenty tubes of any given culture were freeze-dried at any one time, replicate tubes were always available for reference. The initial inoculum into a defined medium was taken from an agar slope by a small loop. Subsequent inoculations into chemically defined media were made either with the small loop or by a drop (0·05 ml.) of a diluted cell suspension of just visible turbidity. The objective in serial subcultivation in chemically defined media was, of course, to eliminate positive growth due to a carry-over of nutrient from the previous tube. With highly potent compounds such as biotin several subcultures were sometimes needed before no growth occurred in a biotin-deficient medium with an organism which proved later to be unable to synthesize biotin. During serial

subcultivation in minimal or nearly minimal media the possibility of selecting organisms which were better synthesizers of essential metabolites not present as nutrients was always borne in mind. A simplified medium arrived at by serial subcultivation in progressively simpler media was always tested for ability to support the growth of the original strain by going back to inocula from an agar slope which itself had been inoculated with a freshly revived desiccate. The latter procedure also acted as a check on the possibility that a contaminated culture had been carried forward. The usual signs of contamination (change in macroscopic and microscopic appearance of the culture, sudden ability to grow in a deficient medium, etc.) were carefully watched for, but with these closely related species could not be relied upon to exclude contamination. In critical cases the organisms obtained growing on a simplified defined medium after serial subculture were re-identified, as a further check.

Determination of nutritional efficacy of defined media

The efficacy of defined media in supporting growth was determined by visual observation of the sets of tubes, with readings at 24 hr. intervals for periods usually up to 3–4 days. The method of study usually took the form of a set of tubes of defined media, which differed from one another by the presence of one or more growth factors or of different N sources, the whole set being inoculated at the same time with the same inoculum. Subsequent visual inspection then showed the relative amounts of growth which occurred in the different tubes of a set of media of different compositions. The relative amounts of growth were scored in such measures as tr. (trace), sl. (slight), ±, +, ++, +++. This way of observing relative amounts of growth was adequate for determining response to various components of the media, since it was being used, in effect, as a qualitative determination of the ability of given substances to satisfy nutrient requirements. No attempt was made to determine optimal, but only minimal or near minimal nutrient requirements. In the present work, too, we were content to find that aneurin, for example, was an adequate nutrient, without going on to find whether the intact molecule of aneurin was essential or whether the vitamin thiazole + pyrimidine, or one of these components, was adequate. That is, in general we have not determined degrees of biosynthetic ability, but have sought to define nutritional requirements in terms of nutritionally adequate known compounds and not necessarily in terms of minimal requirements.

The nutrients examined

The nutritional requirements chiefly examined were:

(*a*) *Sources of nitrogen*. Ammonium ion, certain mixtures of amino-acids, and acid-hydrolysed casein were used. The chief requirements examined were ability to use ammonium ion and if not, ability to use acid-hydrolysed casein, i.e. a collection of most of the naturally occurring amino-acids of protein. The latter was sometimes fortified by addition of tryptophan, cystine (or cysteine) and threonine. Only in a few cases was the casein-hydrolysate replaced by mixtures of known amino-acids; in general we did not study exact amino-acid requirements.

(*b*) *Essential metabolites, mainly of the vitamin B group.* Nutrient requirements satisfied by aneurin, biotin, nicotinic acid, pantothenic acid, pyridoxin, folic acid or riboflavin were examined as a routine. From among these components adequate nutrients were, with few exceptions, found for our strains.

(*c*) *Glucose* (3 %, w/v) was added to all media, unless otherwise mentioned. Other utilizable carbon sources, other than amino-acid mixtures, were not used.

(*d*) *Salt composition and trace elements.* This category of nutrient requirements was not analysed. The usual salts and known traces of various elements were present in the media used and kept relatively constant. The criterion was that growth occurred, although of course not necessarily optimal growth, but enough to make clear the effect of omitting any component from a given medium.

For certain species a special addition of NaCl was required (see below). In many batches of acid-hydrolysed casein medium less complex salt mixtures were added than when using the ammonia basal medium. The acid-hydrolysed caseins were commercial products and were by no means salt-free. Thus, though they added unknown elements to the media, they were adequate for the purposes for which they were intended, namely, as a source of a collection of L-amino-acids in the proportions as in a natural protein, and free at least from growth factors of the vitamin B group. In critical cases special microbiological tests for absence of specific essential metabolites were made; and the casein hydrolysate was replaced in some cases by an amino-acid mixture of known composition.

Composition of media

We used two basal media: (*a*) ammonia basal medium, (*b*) acid-hydrolysed casein basal medium, to which were added sterile solutions of glucose (to final concentration 3 %) and growth factors as required. In some cases mixtures of amino-acids were used instead of ammonia or hydrolysed casein.

Ammonia basal medium. This consisted of: KH_2PO_4, 1·5 g.; $(NH_4)_2HPO_4$, 7·0 g.; $MgSO_4.7H_2O$, 0·5 g.; $CaCl_2.2H_2O$, 0·3 g.; $MnSO_4.4H_2O$, 40 mg.; $FeSO_4.7H_2O$, 2·5 mg.; ammonium molybdate, 2·0 mg. These salts were dissolved together in distilled water (1 l.) and the pH adjusted to pH 7·0. The solution was boiled, filtered and tubed in 5 ml. lots and sterilized by autoclaving for 20 min. at 115°.

The casein basal medium was made from acid-hydrolysed casein vitamin-free (Ashe Laboratories Ltd., Victoria Street, London, S.W. 1, and Allen and Hanbury's Ltd., London, E. 2). Samples were analysed on receipt for moisture, total ash, total nitrogen, Cu and Fe, and used so as to give a total nitrogen content of 0·1 % in the finished medium, which had the following composition: sufficient acid-hydrolysed casein powder; tryptophan, 0·1 g.; cystine, 0·01 g.; K_2HPO_4, 5·0 g.; NaCl, 1·0 g. These components were dissolved in 1 l. distilled water, the pH adjusted to pH 7, boiled, filtered, tubed in 5 ml. lots and sterilized by autoclaving 10 min. at 110°.

Medium 7 AA. Ammonia basal medium was supplemented with the following seven amino-acids in the concentrations shown (in mg./ml.): L-asparagine,

0·04; L-proline, 1·0; L-leucine, 1·5; DL-alanine, 0·35; L-glutamic acid, 0·5; DL-serine, 0·2; DL-methionine, 0·1.

Medium 14AA. Ammonia basal medium was supplemented with the following fourteen amino-acids in the concentrations shown (in mg./ml.): DL-alanine, 0·08; DL-aspartic acid, 0·10; L-arginine HCl, 0·16; L-cysteine HCl, 0·25; L-glutamic acid, 0·50; glycine, 0·08; L-histidine HCl, 0·16; L-leucine, 0·10; DL-methionine 0·16; DL-phenylalanine, 0·20; DL-serine, 0·08; DL-tryptophan, 0·20; L-tyrosine, 0·14; DL-valine, 0·32 (cf. Katznelson & Lochhead, 1947).

Glucose. A 50 % (w/v) solution was sterilized by steaming (100°) for 30 min. on 2 successive days. A volume of 0·3 ml. of this solution was added per 5 ml. basal medium.

Growth factors. Stock solutions were made up and sterilized by autoclaving (20 min. at 115°) except in the case of thermolabile compounds which were sterilized by filtration through Ford S.B. Sterimats, using Pyrex glass filter-holders (Knight & Fildes, 1936). The following final concentrations in media were used: biotin, 1 mμg./ml.; folic acid, 2 mμg./ml.; riboflavin, 0·1 μg./ml.; aneurin, nicotinic acid, pyrodoxin HCl and Ca pantothenate all at 0·5 μg./ml. Stock solutions of fifty times these concentrations were made up and 0·1 ml. added per 5 ml. of basal medium as required.

RESULTS

In reporting our results here 'new isolate' means 'newly isolated strain'; it is part of our object to compare as many properties as possible of such newly isolated strains with those of laboratory strains. The results are recorded species by species, in the order adopted in Bergey's *Manual*, 6th ed. (1948). The whole survey is summarized in Tables 6 and 7. Strain numbers preceded by CN refer to the catalogue number in the Culture Collection at these laboratories; numbers preceded by ATCC are the American Type Culture Collection numbers and those prefaced by NCTC are from the National Collection of Type Cultures (Colindale).

Bacillus subtilis

Fifteen new isolates were studied in detail. Smith *et al.* (1946) considered the following characters as the more important in identifying this species: the morphology, in particular a width of vegetative rod of $<0{\cdot}9\,\mu$.; the growth on glucose agar, glucose nitrate agar, tyrosine agar and potato; positive VP reaction; acid without gas from glucose, arabinose and xylose; hydrolysis of starch, gelatin and casein; the reduction of nitrate to nitrite. They separated this species from *B. pumilus* because *B. pumilus* did not hydrolyse starch or reduce nitrate to nitrite.

Apart from finding that growth on glucose nitrate agar and glucose nutrient agar was of little value in identification, the observations of Smith *et al.* were confirmed. In addition, all these strains were strictly aerobic and did not give a positive Gibson & Abdel-Malek (1945) reaction.

Nutrition. Twenty-six of twenty-seven strains of *B. subtilis* grew at 37° with ammonia as sole source of nitrogen and required no growth factors.

Fifteen were new isolates and twelve were laboratory strains, including two from culture collections (CN 1707 = NCTC no. 2586; CN 2189 = ATCC no. 6633 = N. R. Smith no. 231).

The one exceptional strain (CN 2745) failed to grow with ammonia but grew when given proline, leucine, alanine, serine and asparagine. This amino-acid mixture was not minimal however; omission of asparagine delayed growth for 24 hr. and omission of alanine in presence of asparagine prevented growth, at least up to 96 hr. Serine appeared to be an absolute requirement in the above mixture.

Bacillus subtilis *var.* aterrimus *and* Bacillus subtilis *var.* niger

The characters used to differentiate these two varieties are described in Smith *et al.* (1946; cf. Bergey, 1948, p. 706). The *aterrimus* variety forms a black pigment on carbohydrate medium, the variety *niger* only on media containing tyrosine.

The two strains of *aterrimus* and the one of *niger* examined grew on the ammonia basal medium and needed no added growth factors. The strain of *aterrimus*, CN 2743, was identified as such in these Laboratories, and was among a collection of laboratory strains received as *B. subtilis*. The other strain (CN 2192) of *aterrimus* was ATCC no. 6464 (= N. R. Smith no. 275). The single strain of *niger* was likewise from the ATCC being no. 1972 (= N. R. Smith no. 650). Nutritionally, therefore, these three strains were indistinguishable from typical strains of *B. subtilis*.

Bacillus licheniformis

Gibson (1944) showed that it was possible to differentiate certain strains of a *subtilis*-like organism sharply from *B. subtilis* proper, that the correct designation for what appeared to be a distinct species was the new combination *B. licheniformis* (Weigmann) Gibson, and that among the most important characters of *B. licheniformis*, differentiating it from *B. subtilis*, were the ability to grow anaerobically and to give a positive Gibson & Abdel-Malek (1945) reaction.

The metabolism of strains of *B. licheniformis* has been considerably studied because of the striking and characteristic products formed from glucose under anaerobic conditions, namely, 2, 3-butanediol and glycerol in approximately equimolar concentrations. The relatively high concentration of glycerol formed is rare in bacterial metabolism. Organisms with this characteristic metabolism were used under the name of *B. subtilis*, Ford's type or Ford's strain, and compared with *B. subtilis*, Marburg type, for example by Blackwood, Neish, Brown & Ledingham (1947). The typical metabolism of the Ford type strains (= *B. licheniformis*) was quite different from that of the Marburg type strains (= *B. subtilis*). These authors supported the suggestion of Gibson that these Ford type strains deserved separate species rank. Smith *et al.* (1946) did not study any organisms under the name *B. licheniformis* nor did they comment on the characteristics which might entitle this organism to specific rank. Bergey (1948) refers to *B. licheniformis* only in the appendix to the genus; but it is

clear from the dates of the references there given that the subsequent strong evidence accumulated by Gibson (1944) and by Blackwood *et al.* (1947) appeared after the relevant portion of Bergey's *Manual* had been prepared.

We studied twelve strains of *B. licheniformis* having the characters described by Gibson (1944); four of these strains were newly isolated and eight were from the Microbiological Research Department (Ministry of Supply), Porton. In view of the differential importance of the ability to grow strictly anaerobically, which these twelve strains possessed, and because of the unusual anaerobic dissimilation of glucose, we support the assignment of this organism to specific rank.

Nutrition. All twelve strains grew with ammonia as sole source of nitrogen and required no growth factors. Thus nutritionally these strains were indistinguishable from typical strains of *B. subtilis* so far as nitrogen source and non-requirement of growth factors was concerned. Since, however, *licheniformis* strains grow anaerobically in ordinary media (*subtilis* strains cannot) and have a characteristic anaerobic glucose dissimilation, nutritional differences in utilizable carbon and energy sources could undoubtedly be shown.

Bacillus pumilus

Twelve new isolates were studied in detail. Smith *et al.* (1946) considered the following characters to be important in identifying this species: the morphology, including a width of vegetative rod of $<0{\cdot}9\,\mu$.; positive VP reaction; production of acid from glucose, arabinose and xylose; failure to hydrolyse starch; failure to reduce nitrate.

These properties were observed with all our new isolates except that a few failed to ferment arabinose or xylose. All these strains grew only aerobically, did not produce urease and grew in 4·0 % NaCl broth and on nutrient agar at pH 6·0. All strains produced a 'restricted' egg-yolk reaction, in contradistinction to *B. subtilis*, which was consistently negative.

Nutrition. Twenty-one strains were examined (eleven new isolates and ten laboratory strains which included ATCC no. 7061 = N. R. Smith no. 272 = CN 2200). With two exceptions these strains grew on ammonia basal medium + DL-biotin. The exceptions were CN 807, which would not grow with ammonia + biotin but did so with glutamic acid + biotin, and CN 2746, which similarly would not grow with ammonia but did so when given asparagine + leucine + biotin. These two strains were thus like the other nineteen in having a nutrient requirement satisfied by biotin; but their ability in amino-acid synthesis was more restricted, since they could not use only ammonia for that purpose (Table 1).

The specificity of the biotin requirement was examined only with strains CN 787, 2731, 2732, 2733, 2735 and 2736. All strains used DL-biotin and DL-O-heterobiotin. But strain CN 2733 could not utilize DL-desthiobiotin, whereas all the others of this group of strains did so. Thus CN 2733 is a strain in which the biosynthesis of biotin is blocked at least at the conversion of desthiobiotin to biotin, whereas the block occurs earlier in the other strains. The utilization of other possible precursors in biotin synthesis is being further examined.

Table 1. *Comparison of nutritional and other characters of* Bacillus subtilis, B. licheniformis, B. pumilus *and* B. subtilis/pumilus *intermediates*

Organism	No. of strains	Anaerobic growth	Gibson & Abdel-Malek reaction	Nitrate reduction	Starch hydrolysis	Nutrient requirements: Ammonia	Nutrient requirements: Amino-acids	Nutrient requirements: Biotin
B. subtilis	26	−	−	+	+	+	−	−
	1	−	−	+	+	−	+	−
B. licheniformis	12	+	+	+	+	+	−	−
B. pumilus	19	−	−	−	−	+	−	+
	2	−	−	−	−	−	+	+
Intermediates:								
CN 1582	.	−	−	−	+	+	−	−
CN 1583	.	−	−	−	+	+	−	−
CN 2499	.	−	−	−	+	+	−	+
CN 2549	.	−	−	−	+	+	−	+

One of these strains (CN 787) was received labelled '*B. subtilis*'. When it failed to grow on ammonia media without biotin, but grew when biotin was added, the culture was re-examined and found to be a typical *pumilus*, being unable to hydrolyse starch or reduce nitrate. Thus the nutritional character drew attention to an incorrect identification.

Bacillus subtilis/pumilus *intermediates*

Four cultures, all new isolates, which were intermediate in character between *B. subtilis* and *B. pumilus* were examined. The differentiation of *B. subtilis* from *B. pumilus* at present depends on the hydrolysis of starch and the reduction of nitrate by *B. subtilis*, whereas *B. pumilus* does neither (Smith *et al.* 1946). These four intermediate strains all hydrolysed starch, like *B. subtilis*, but, like *B. pumilus*, did not reduce nitrate. The egg-yolk reactions were negative. Two of these strains (CN 1581 and 1582) grew with ammonia alone, i.e. like *B. subtilis*, while two (CN 2499 and 2459) grew only on ammonia basal + biotin, i.e. they were like typical *B. pumilus* strains in nutritional requirements.

Thus nutritionally these 'intermediates' corresponded with the typical nutrition of one or other of the two relevant species (see Table 1).

Bacillus coagulans

Fifteen new isolates were studied in detail. Smith *et al.* (1946) considered the following characters to be the more important in identifying this species: better growth at 45° than at 37° or 28°; positive VP reaction; starch hydrolysed; no hydrolysis of gelatin; weak hydrolysis of casein; heavy growth on glucose broth with a resultant pH of about 4·0 after 7 days and with the rapid evolution of gas with metallic iron. These characters were found in all fifteen new isolates, although the better growth observed at 45° might be a reflexion of our method of isolating the organisms from the soil. Smith *et al.* state that this organism usually produces acid from arabinose but not from xylose. With

our new isolates this property was very variable; ten produced acid from both arabinose and xylose and one did not produce acid from either sugar.

All the strains grew anaerobically, did not produce urease, and did not produce either type of egg-yolk reaction.

Nutrition. Sixteen strains were examined; one was a laboratory strain (ATCC no. 7050 = N. R. Smith no. 609 = CN 2202), the remainder being newly isolated. A temperature of 45° was found preferable for these organisms. All strains grew well in the casein basal medium supplemented with aneurin and biotin; the ammonia basal medium could not replace the casein medium. Thus all strains could not use ammonia as sole source of nitrogen and required amino-acids. There was some evidence that not all strains required both aneurin and biotin, but that a few synthesized one or other of these compounds, or variants with these abilities were rather easily selected during subcultivation.

There was no evidence for any growth factor requirements other than aneurin and biotin. This point was examined in some detail, since Cleverdon *et al.* (1949*a*) reported a strain of *B. coagulans* (N. R. Smith, no. 27) which grew when aneurin, biotin and nicotinic acid were added to a basal casein-hydrolysate medium, but not in the absence of any one of these three essential metabolites. Our casein basal medium was tested for nicotinic acid (or equivalent material) by attempting to grow a strain of *Proteus vulgaris* in it. This organism did not grow until the medium was supplemented by nicotinic acid. The medium therefore was effectively free from nicotinic acid. By the criterion of ability to grow on this casein medium supplemented by aneurin and biotin but without nicotinic acid, it can therefore be said that none of the fifteen newly isolated strains of *B. coagulans* examined, nor ATCC no. 7150, required nicotinic acid as a nutrient.

Bacillus firmus

Only three new isolates were obtained from soil. These were studied in detail, together with two strains from collections. Smith *et al.* (1946) considered the more important differential characters to be: morphology; no growth at pH 6·0; character of the growth on nutrient agar and glucose nutrient agar; failure to utilize inorganic nitrogen; hydrolysis of starch, gelatin and casein; failure to produce urease.

Our five strains had these characters. Smith *et al.*, however, felt that this organism was difficult to classify since it has a morphology intermediate between that of the *B. subtilis* group and the *B. circulans/brevis* group, and had some of the physiological characteristics of *B. brevis* and *B. sphaericus*. In only one of our five strains were the sporangia slightly swollen, and we feel that the organism is, morphologically, clearly in group 1. The physiological activity of these strains also was quite different from that of *B. sphaericus*, which is relatively inert. *B. firmus* ferments glucose, hydrolyses starch, gelatin and casein and sometimes reduces nitrate, while *B. sphaericus* only hydrolyses gelatin. We also found *B. firmus* unable to grow anaerobically, thus separating it from the *B. circulans* group.

Nutrition. A preliminary examination indicated that the nutritional requirements were complex.

Bacillus lentus

Two strains of *B. lentus* were isolated from soil; these were studied, together with one laboratory strain (Gibson's no. 165). Smith *et al.* (1946) studied one strain and provisionally allocated the organism to morphological group 1, near to *B. firmus*. These three strains of *B. lentus* differed from *B. firmus* in producing urease, not hydrolysing gelatin or casein, and not reducing nitrate; they were not able to grow anaerobically. Preliminary experiments indicated complex nutritional requirements.

Bacillus firmus, Bacillus lentus *and related organisms*

Of eleven new isolates which were clearly in the *B. firmus-B. lentus* group, five conformed to the type description of one or other species (see above), but six could not be classified. They differed from either of the two species in more than one character such as hydrolysis of starch, casein or gelatin and reduction of nitrate. This represents a high proportion of isolates which could not be classified and is different from our experience with other species.

Bacillus megatherium

Twelve new isolates were examined in detail. Smith *et al.* (1946) considered the following characters as the more important in identifying this species: the well-known morphological appearance of Gram-stained preparations, in particular a width of vegetative rod greater than $0{\cdot}9\,\mu$.; negative VP reaction; production of acid without gas from glucose, arabinose and usually xylose; hydrolysis of starch; heavy growth on glucose nitrate agar.

In our experience growth on glucose nitrate agar was of little diagnostic value, strain variation being as great as species variation. Four of our twelve new isolates failed to grow on glucose nitrate agar, and two strains failed to hydrolyse starch; otherwise they agreed with the above characters.

Two additional tests were of great value in differentiating *B. megatherium* from *B. cereus*. *B. megatherium* was unable to grow anaerobically and did not produce any egg-yolk reaction, whereas *B. cereus* gives positive reactions in these tests (Table 2).

Nutrition. All eleven strains examined (eight new isolates and three laboratory strains including ATCC no. 8245 = N. R. Smith no. 234 = CN 2193) grew on the ammonia basal medium alone; no growth factors were needed (Table 2).

Bacillus cereus

Twelve new isolates were examined in detail. Smith *et al.* (1946) considered the following characters as the more important in identifying this species: the morphology, including a width of vegetative rod greater than $0{\cdot}9\,\mu$.; colonial appearance; positive VP reaction; production of acid from glucose but not from arabinose and xylose; hydrolysis of starch.

Table 2. *Nutritional and other characters of* Bacillus megatherium, B. cereus *and intermediates*

(+ = positive reaction; (+) = variable reaction.)

Organism	Voges-Proskauer reaction	Acid from arabinose and xylose	Egg-yolk reaction (lecithinase)	Anaerobic growth	Nutrition: Ammonia used	Nutrition: Amino-acids required
B. megatherium, 11 strains	−	+	−	−	+	−
B. cereus, 13 strains	+	−	+	+	.	+
B. cereus var. *mycoides*, 10 strains	+	−	+	+	.	+
Intermediates:						
CN 736	+(4 days)	+	−	−	+	−
CN 2496	(+)	−	−	−	+	−
CN 2481	+feeble	+	+	+	.	+
CN 2502	+	+	+	+	.	+
CN 2509	+	+	+	+	.	+
CN 2513	+	+	+	+	.	+

These characters were found in all our strains and furthermore all the *B. cereus* strains, in contradistinction to *B. megatherium*, grew anaerobically and produced the characteristic egg-yolk reaction, which has been shown by McGaughey & Chu (1948) to be due to a lecithinase C.

Nutrition. Thirteen strains were examined (eleven new isolates and two laboratory strains including ATCC no. 6630 = N. R. Smith no. 305 = CN 2194). None of them utilized ammonia as sole nitrogen source. All grew on the hydrolysed casein medium, which could be replaced by certain mixtures of amino-acids. There was no evidence of any other nutrient requirements (i.e. for growth factors). Several strains were grown with mixtures of amino-acids. Three strains (CN 2194, 2503 and 2504) grew on a mixture of seven amino-acids (medium 7AA), namely, L-asparagine, DL-proline, L-leucine, DL-alanine, L-glutamic acid, DL-serine and DL-methionine.

Eight strains (CN 2484, 2501, 2505, 2506, 2507, 2508, 2511, 2512 and 2810) grew on a mixture containing fourteen amino-acids (medium 14AA), namely, DL-alanine, DL-aspartic acid, L-arginine, L-glutamic acid, glycine, L-histidine, L-leucine, DL-methionine, DL-phenylalanine, DL-serine, DL-tryptophan, L-tyrosine, L-cysteine and DL-valine.

The requirements for specific amino-acids in these mixtures were not further analysed. Ability to grow on these mixtures, however, substantiates the conclusion that none of the strains examined required any of the vitamin B group of essential metabolites.

Among the laboratory strains one (now CN 2810), originally labelled *B. subtilis*, proved unable to grow on ammonia medium and required amino-acids, which made it unlike characteristic *subtilis* strains. Other diagnostic tests then proved it to be *B. cereus*, producing, for example, a positive typical egg-yolk reaction. Here again apparently anomalous nutritional requirements led to a correct identification. The results with all strains are summarized in Table 2.

Bacillus cereus *var.* mycoides

Twelve new isolates were examined in detail. They differed from *B. cereus* only in the rhizoid nature of the colony on nutrient agar. With our strains *B. cereus* showed a slight preference for growth at 37° as against 28°, whereas the *B. cereus* var. *mycoides* strains showed a marked preference for 28° and grew poorly or not at all at 37°.

B. cereus var. *mycoides* when incubated at room temperature in nutrient broth and then plated on nutrient agar produced a number of colonies indistinguishable from *B. cereus*. One strain was examined in more detail. The original culture of *B. cereus* var. *mycoides* gave typical colonies, grew well at 28° and did not grow at 37°, whereas the *cereus*-like variant obtained from it grew slightly better at 37° than at 28°. On subcultivation in casein basal medium or a 14-amino-acid mixture (medium 14AA) the typical growth of var. *mycoides*, as a submerged pellicle which remained intact on agitating the culture tubes, changed with some strains to a diffuse growth like that of typical strains of *B. cereus* under the same conditions. The change from the *mycoides* to the *cereus* characters was not further studied. Nutritionally (see below) and in all other respects except the typical colonial growth on plates and in liquid media, these *mycoides* strains were identical with the *cereus* strains. The opinion of Smith *et al.* (1946) that '*B. mycoides*' is a rhizoid variant of *B. cereus* is supported by our observations.

Nutrition. All ten strains examined (including ATCC no. 6463 = N. R. Smith no. 306 = CN 2195 and nine new isolates) did not grow on the ammonia basal medium but grew in the casein basal medium without added growth factors. Some strains were grown on the 14-amino-acid mixture (medium 14AA) but the amino-acid requirements were not further analysed. Thus the nutrient requirements were satisfied by the media suitable for *B. cereus* (see Table 2).

Bacillus megatherium/cereus *intermediates*

A number of new isolates were classified as *B. megatherium/cereus* intermediates since they were aberrant either in the VP reaction or in the fermentation of arabinose and xylose. These intermediates fell into two groups: those which did not grow anaerobically and did not produce any egg-yolk reaction (i.e. were like *megatherium*) and those which grew anaerobically and produced a typical *cereus*-like egg-yolk reaction. It seemed rational therefore to classify these intermediates as *B. megatherium* or *B. cereus* respectively. We did not find a strain which grew anaerobically and failed to produce the typical *cereus*-like egg-yolk reaction, or one which did not grow anaerobically but did produce a *cereus*-like egg-yolk reaction. The specific nutritional requirements supported this separation (see Table 2).

Nutrition. Six strains (five new isolates) which were classified as *megatherium/cereus* intermediates had the nutritional characters summarized in Table 2, two being like *B. megatherium* in using ammonia, and the other four requiring amino-acids like *B. cereus*.

Bacillus polymyxa

Twelve new isolates from soil were studied in detail. Smith *et al.* (1946) considered the following characters as the more important in identifying this species: the morphology; positive VP reaction; fermentation of glucose, arabinose and xylose, with the production of gas; hydrolysis of starch. These characters were found with our new isolates. Additional characteristics of the organism were an ability to grow anaerobically and to give a positive Gibson & Abdel-Malek test.

Nutrition. Katznelson & Lochhead (1944) examined the nutrient requirements of eighty-two strains of *B. polymyxa* and found that all grew on an ammonia basal medium plus biotin. The thirteen newly isolated strains we examined showed these same nutrient requirements. Since we were mainly concerned with minimal requirements we did not examine the adjuvant effect of other nitrogen sources and growth factors as did Katznelson & Lochhead. We did not find an authentic *B. polymyxa*, i.e. as characterized according to Smith *et al.* (1946), which grew in ammonia basal medium without biotin, nor one which did not grow on the ammonia medium when it was supplemented with biotin. We have not yet examined the replacement of biotin by possible precursors in its biosynthesis, nor the effect of oleic acid, etc. Ability to use ammonia + biotin appears to be a typical character of *B. polymyxa*.

The Bacillus macerans-circulans-alvei *group*

The organisms classified under these names in the work of Smith *et al.* (1946) are less clearly differentiated, or are differentiated by less striking characters, than are many other species in the genus. We encountered many strains with characters intermediate between pairs of species, as defined. This holds for the nutritional patterns as well as for the other characters. But often the nutritional patterns within groups of intermediates did not correspond with a subdivision based on some other character, unlike the *subtilis/pumilus* and *megatherium/cereus* intermediates, where the respective nutritional patterns were regularly associated with other species characters. For this reason, therefore, the general bacteriological findings and the nutritional findings with the aberrant and intermediate strains are reported separately in Tables 3 and 4. It remains to be seen whether this non-correspondence of physiological and nutritional characters in the intermediate strains and the greater heterogeneity of nutritional requirements of typical strains within a given species, is really the reflexion of imperfect differentiation or is to be considered as a natural feature characterizing the group as a whole.

In the following sections the strains are described as much as possible under species groupings, all strains under a given species name agreeing with the species description. The exceptional strains are described with the nearest species or as frank intermediates.

Table 3. *The relationship between* Bacillus macerans, B. circulans, B. alvei, B. laterosporus *and intermediates*

(All these organisms belong to the Smith, Gordon & Clark morphological group 2 and are facultative aerobes. They are differentiated by the reactions shown below.)

Species	Acid or gas from			Voges-Proskauer reaction	Hydrolysis of		
	Glucose	Arabinose	Xylose		Starch	Casein	Gelatin
B. macerans	+gas	+gas	+gas	−	+	−	+
B. circulans	+	+	+	−	+	−	+
B. alvei	+	−	−	+	+	+	+
B. laterosporus	+	−	−	−	−	±	+
Intermediate strains:							
CN 2628, 2629, 2718	+	+	+	−	+	−	−
CN 2755, 2756	+	−	−	−	+	−	−
CN 2807	+	+	+	−	+	+	+
CN 2482	+	−	−	+	−	−	−
CN 2589, 2620	+	+	+	+	+	−	−

Table 4. *Nutritional requirements of* Bacillus macerans, B. circulans, B. alvei *strains and intermediates*

	Nutrients utilized			
	Ammonia	Amino-acids	Aneurin	Biotin
B. macerans:				
CN 1013 (NCTC no. 3223); CN 1014 (NCTC no. 4743); CN 2204 (ATCC no. 7068); CN 2614 (smooth variant of CN 1014); CN 2729 (new isolate)	+	−	+	+
B. alvei:				
CN 2198 (ATCC no. 6344); CN 2771, 2772	−	+	+	−
CN 2482 (aberrant)	−	+	+	+
CN 2773	Unidentified			
B. circulans:				
CN 2201 (ATCC no. 7049); CN 2526, 2621, 2720, 2721, 2722, 2848, 2849, 2905, 2923, 2924, 2925	−	+	+	+
CN 1609, 2628, 2718, 2719, 2723	Unidentified			
circulans/alvei intermediates:				
CN 1761, 2620, 2755	−	+	+	+
CN 2588, 2589, 2629, 2756	Unidentified			

Bacillus macerans

Only one strain of *B. macerans* was isolated from soil and was studied with four laboratory strains. The description of Smith *et al.* (1946) was confirmed with these five strains. We also noted the ability of these strains to grow anaerobically and to give a weak positive Gibson & Abdel-Malek test. We also observed, with our technique, considerable irregularity in the production of gas from sugars.

Nutrition. Katznelson (1944) compared the nutritional requirements of *B. polymyxa*, *B. macerans* and one strain of '*B. acetoaethylicus*' with a view to their differentiation; all his eighty-two strains of *B. polymyxa* grew on ammonia + biotin (cf. Katznelson & Lochhead, 1944) and the three strains of *B. macerans* and the one strain of '*B. acetoaethylicus*' which he examined grew only with the further addition of aneurin. In Bergey's *Manual* (1948), following Porter, McClesky & Levine (1937), *B. acetoaethylicum* (originally described by Northrop, Ashe & Senior, 1919) is regarded as a synonym for *B. macerans*.

Only one of our five strains was newly isolated, and one (CN 2614) was a smooth variant derived from CN 1014 (NCTC 4743) which showed some difference from the parent organism as regarded sugar fermentations. The four laboratory strains and the new isolate all grew on ammonia + biotin + aneurin but not on the ammonia medium with aneurin or biotin alone. This is in agreement with Katznelson (1944). These five strains were quite distinct nutritionally from all other strains in this group of species in being able to utilize the ammonia basal medium and in not requiring any amino-acids.

Bacillus circulans

Fifteen new isolates were studied in detail. The chief diagnostic characters of *B. circulans* are: negative VP reaction; fermentation of glucose and usually arabinose and xylose; hydrolysis of starch and gelatin but not casein. These characters, described by Smith *et al.* (1946), were possessed by all our strains. In addition they were able to grow anaerobically, did not give a positive Gibson & Abdel-Malek test or any egg-yolk reaction. The ability to grow anaerobically separates *B. circulans* from the *B. firmus-B. lentus* group and from *B. brevis*. The failure to give a restricted egg-yolk reaction separates it from *B. alvei*. However, the close relationship between *B. macerans*, *B. circulans* and *B. alvei*, commented on by Smith *et al.* (1946), was evident and many intermediate types were isolated.

Nutrition. Many strains of *B. circulans* were difficult to grow, and we are not satisfied that we have yet found the best basal medium for revealing the nutritional requirements. Nevertheless, the positive responses to certain essential metabolites may be considered valid.

Eighteen strains classified as *B. circulans* according to Smith *et al.* (1946) were examined; these included two laboratory strains (one being ATCC no. 7049 = N. R. Smith no. 358 = CN 2201), the remaining sixteen being newly isolated.

The nutritional requirements of twelve of these strains were satisfied by casein basal medium + biotin + aneurin; but the growth of six strains was irregular on this medium, nor was it improved by the addition of riboflavin, pyridoxin, folic acid, pantothenic acid, or nicotinic acid, either singly or in combination. Whether the bad and irregular growth of these strains was due to their requiring some biosynthetically 'higher' derivative of one of these substances (e.g. coenzyme I instead of nicotinic acid, pyridoxamine or pyridoxal instead of pyridoxin), or for other specific nutrients, or whether some

physico-chemical requirement (e.g. salt composition or concentration) was not satisfied, has not yet been determined. A few strains, e.g. CN 2848 and 2925, were made to grow with ammonia instead of the casein hydrolysate, but these ammonia-utilizing cultures had probably been selected during subcultivation; the parent cultures required amino-acids. Table 4 summarizes the results with *B. circulans*.

Bacillus alvei

Only a few strains of *B. alvei* were studied, namely one laboratory strain, two strains from honeycombs infected with European foul-brood (received from Dr E. Schreiner), and one isolated by us from soil. As described by Smith *et al.* (1946) this species is distinguished from *B. circulans* by: positive VP reaction; failure to ferment arabinose or xylose; ability to hydrolyse casein. The four strains we examined produced a restricted type of egg-yolk reaction and grew anaerobically.

Nutrition. Katznelson & Lochhead (1947) found that ten strains of *B. alvei* required aneurin when grown in a hydrolysed casein medium or in a mixture of amino-acids. *B. para-alvei*, isolated from bee larvae affected with 'para-foulbrood' disease (Burnside, 1932; Burnside & Foster, 1935), was considered by Smith *et al.* (1946) to be identical with *B. alvei*. Three strains examined by Katznelson & Lochhead (1947) grew in certain amino-acid mixtures without added aneurin, but not in the acid-hydrolysed casein medium without aneurin. Thus the *B. para-alvei* and *B. alvei* strains of Katznelson & Lochhead differed nutritionally and fell into two groups distinguished by the requirement of *B. alvei* for aneurin when growing on the hydrolysed casein medium. Later Katznelson (1947) found that *B. para-alvei* grew abundantly in mixtures of fifteen to eighteen amino-acids with aneurin and moderately in the same amino-acid mixtures without aneurin. Certain omissions from the amino-acid mixtures greatly affected growth in the absence of aneurin; omission of phenylalanine, valine and *iso*leucine much decreased the growth and omission of cystine stopped it completely. The cystine could be replaced by certain sulphur-containing substances, e.g. glutathione, homocystine and homocysteine, thiol-acetate or thiosulphate, but not by methionine, thiourea, thiouracil or sulphite. It was suggested that perhaps the active sulphur-containing compounds were utilized as precursors in the biosynthesis of aneurin.

We examined only five strains of *B. alvei* (ATCC no. 6344 = N. R. Smith no. 662 = CN 2198), three new isolates, and one other new isolate (CN 2482) which was aberrant (see Table 3). These strains, with the exception of CN 2482 and 2773, grew on the casein medium when aneurin was added but not without it. Ammonia could not be substituted for the hydrolysed casein but growth was obtained on certain mixtures of amino-acids, again only in the presence of aneurin, biotin not being required as a nutrient. The aberrant strain CN 2482 required both biotin and aneurin as supplements to the casein basal medium. In this respect, therefore, this organism resembled some *circulans* strains from which, however, it was distinct on other grounds. The remaining strain, CN 2773, appeared to have more complex requirements than the other *alvei* strains and was not grown satisfactorily.

Our results (see Table 4), therefore, as far as they go, agree fairly well with those of Katznelson & Lochhead; we have not yet investigated the specificity of the aneurin requirements.

Thus the well-defined *B. alvei* strains differed from *B. circulans* in that the former did not require biotin as a nutrient, whereas the majority of the *B. circulans* strains required at least aneurin and biotin, and some strains had even more complex requirements.

Bacillus macerans-circulans-alvei *group; aberrant and intermediate strains*

B. macerans is distinguished from *B. circulans* by its ability to produce acid and gas from sugars, whereas *B. circulans* only produces acid (Table 3). We examined three strains of *B. macerans* from culture collections, i.e. CN 1013, 1014 and 2204 (=ATCC no. 7068). Peptone water was the basal medium used. Strain CN 1013 produced acid and gas from glucose, lactose, sucrose, mannitol, arabinose, xylose, rhamnose, raffinose, salicin, dextrin, fructose and galactose, but acid only from maltose. Strain CN 1014 differed from CN 1013 in producing acid and gas from maltose but acid only from arabinose. Strain CN 2204 differed from CN 1013 in producing acid and gas from sucrose, mannitol and raffinose but acid only from the other sugars. From strain CN 1014, which was mainly rough, a smooth variant (CN 2614) was selected which differed from the parent strain in producing acid only from glucose, lactose, sucrose, raffinose, salicin and dextrin; acid and gas were produced from the remaining sugars. A strain newly isolated from soil (CN 2729) produced acid and gas from lactose, mannitol, xylose, inulin, fructose, raffinose, arabinose, rhamnose, trehalose, and dextrin but not from glucose, sucrose, maltose or salicin.

All these strains, however, were like *B. macerans* in their nutritional requirements (see p. 527). However, one new isolate (CN 2629) from soil gave reactions similar to *B. macerans* or *B. circulans* but produced acid and gas from salicin and glycerol only; with the other sugars only acid was produced; this strain nutritionally was not a *macerans* (see Tables 3 and 4).

The foregoing results suggest that the fermentation of sugars with the production of gas is not only variable in the *macerans* group, but strains which are not *B. macerans* and are more nearly related to *B. circulans* may produce acid and gas from some sugars.

Some emphasis has been placed on the hydrolysis of casein and gelatin as characterizing *B. circulans*. In Table 3 four strains (CN 2628, 2629, 2718 and 2807) are classified as intermediates, since they either did not hydrolyse casein or gelatin, or hydrolysed both casein and gelatin. However, we feel that these strains should be properly classified as *B. circulans*, and the ability to hydrolyse gelatin and casein regarded as variable with this species.

We also found a number of strains, otherwise like *B. circulans*, which fermented neither arabinose or xylose; they also were classified as *B. circulans*. Also, as shown in Table 3, two strains (CN 2755 and 2756) were isolated which did not produce acid from arabinose or xylose. These strains, together with

those strains (CN 2589 and 2620) which were VP positive and fermented both arabinose and xylose, were classified as *B. circulans/alvei* intermediates. One strain (CN 2482) was isolated which was similar to *B. alvei*, except that it failed to hydrolyse starch, casein or gelatin; it was nutritionally aberrant also (see Table 3).

Nutrition. Six strains (five newly isolated) which were intermediates in properties between *circulans* and *alvei* were examined. Three (CN 2588, 2589 and 2756) were difficult to grow on defined media and nothing can be said about them. The remaining three strains (CN 1761, 2620 and 2755) grew on casein basal medium + aneurin + biotin. One of them (CN 2755) grew in ammonia + aneurin + biotin after prolonged subcultivation, probably owing to selection of an amino-acid synthesizer; the parent culture required hydrolysed casein. Thus three of these intermediate strains resemble *B. circulans* nutritionally, while the other three probably have more complex nutrient requirements and are thus not like *alvei* strains. The results are summarized in Table 4.

It must be emphasized that the *macerans-circulans-alvei* group is outstanding in the genus for the number of aberrant and intermediate strains encountered.

Bacillus brevis

Seven new isolates were studied in detail. Smith *et al.* (1946) considered the following characters as the more important in identifying this species: morphology; production of an alkaline pH in glucose broth after 7 days' incubation; negative VP reaction; production of acid from glucose and sometimes from arabinose and xylose; no hydrolysis of starch; hydrolysis of gelatin and casein. All our strains had these properties (with two minor exceptions), would not grow anaerobically, thus distinguishing them from the *circulans* group, and all produced urease. Smith *et al.* use the utilization of citrate in their Key in separating *B. brevis* from *B. laterosporus*, although they mention it is a variable character. We found the utilization of citrate variable in the strains of *B. brevis* we examined.

Two new isolates from soil (CN 2833 and 2932) were similar to *B. brevis* except that the pH in glucose broth after 7 days was less than 8·0. Both grew aerobically only, and were therefore considered *B. brevis*.

Nutrition. Ten strains were examined (nine newly isolated and strain ATCC no. 8185 = N. R. Smith no. 751 = CN 2203). Of the nine new isolates two (CN 2833 and 2932) were slightly aberrant (see above). All strains, with the exception of the slightly aberrant CN 2932, grew on the casein basal medium without added growth factors. Strain CN 2932, however, was also nutritionally aberrant growing only when aneurin, biotin and nicotinic acid were added to the casein basal medium. Thus the ATCC strain and eight new isolates were homogeneous in nutritional requirements; only one strain, CN 2932, was different and that markedly. A nutritional requirement satisfied by nicotinic acid has been observed among mesophilic organisms of the genus *Bacillus* only for some strains of *B. pasteurii* (see p. 532) and for one strain of *B. coagulans* (Cleverdon *et al.* 1949*a*), and is thus apparently very unusual. Cleverdon *et al.* (1949*b*), however, found two groups of stenothermophilic (55–65°) strains of

Bacillus which required nicotinic acid. Apart from its peculiar nutritional requirements CN 2932 clearly belongs more to *B. brevis* than to any other species.

Bacillus sphaericus *and* Bacillus sphaericus *var.* fusiformis

Twelve new isolates which were considered typical *B. sphaericus* were isolated from soil and studied in detail. Smith *et al.* (1946) gave the following characters as the more important in identifying this species; the morphology, including bulging of the sporangium by a thick-walled spherical spore; growth on nutrient agar at pH 7·4 and 6·0; growth in 4·0 % NaCl broth; hydrolysis of gelatin; negative VP reaction; no fermentation of sugars; no reduction of nitrate; no hydrolysis of starch of casein. All our twelve new isolates conformed to these characters.

Smith *et al.* described three varieties which differed from *B. sphaericus* as follows: *B. sphaericus* var. *rotans*: no growth on nutrient agar at pH 6·0 or in 4·0 % NaCl broth; maximum temperature for growth 35°; *B. sphaericus* var. *fusiformis*: produces urease; *B. sphaericus* var. *loehnisii*: no growth at pH 6·0 and produces urease.

We did not isolate any strains of *B. sphaericus* var. *rotans*, but twelve new isolates each of *B. sphaericus* var. *fusiformis* and *B. sphaericus* var. *loehnisii* were obtained from soil. These strains conformed to the descriptions given by Smith *et al.* (1946); they also failed to grow anaerobically.

Nutrition. Only strains of *B. sphaericus* and *B. sphaericus* var. *fusiformis* were examined critically, and the results with these two organisms are conveniently reported together. Hitherto their differentiation has rested upon the absence of urease from *B. sphaericus* and its presence in *B. sphaericus* var. *fusiformis*. We examined nine strains of *B. sphaericus* (eight new isolates and ATCC no. 10208 = N. R. Smith no. 966 = CN 2205), and ten strains of *B. sphaericus* var. *fusiformis* (eight new isolates and two laboratory strains, one being ATCC no. 7055 = CN 2209). The ten strains of *B. sphaericus* var. *fusiformis* were uniform in that they would not grow on the casein basal medium in the absence of aneurin and biotin, but grew when both were added, neither being effective alone.

On the other hand, six out of nine strains of *B. sphaericus* grew on casein basal medium with added aneurin alone. The remaining three strains of *B. sphaericus* (CN 2485, 2546 and 1652) grew with aneurin + biotin on the casein basal medium, but not with either of these substances singly, i.e. these three strains were nutritionally like the strains of *fusiformis*. They were repeatedly tested for urease but were always negative, i.e. they had the *sphaericus* diagnostic character. It was possible to replace the hydrolysed casein by a mixture of seven amino-acids (medium 7 AA) for many of the strains of *B. sphaericus* and *B. sphaericus* var. *fusiformis*; medium 14 AA was adequate for all strains of *B. sphaericus* and *B. sphaericus* var. *fusiformis* with one exception. Strains differed in the dispensability of certain amino-acids but the mixture of fourteen amino-acids was adequate for most of them.

Only one strain each of *B. sphaericus* var. *loehnisii* and var. *rotans*

were examined; their nutrition appeared to be more complex than that of *B. sphaericus* var. *fusiformis*.

Bacillus pasteurii

Twelve new isolates of *B. pasteurii* were isolated from soil. Smith *et al.* (1946) considered the following characters as the more important in identifying *B. pasteurii*: the morphology, including bulging of the sporangium by a thick-walled spherical spore; inability to grow on nutrient agar; growth on nutrient agar containing 2·0 % urea; the formation of urease; reduction of nitrate to nitrite; growth in 4·0 % NaCl broth (with 1·0 % urea); hydrolysis of gelatin but not of starch; failure to ferment sugars. This description was confirmed with all the twelve new isolates.

With most strains, after 4–7 days in the anaerobic jar, many tiny colonies (diameter *c.* 0·1 mm. or less) were observed.

Nutrition. The work on the differentiation and classification of organisms of the *B. pasteurii* group by Gibson included some studies of nutritional requirements (Gibson, 1934*a*, *b*; 1935*a*, *b*). We examined ten strains of *B. pasteurii* as defined by Smith *et al.* (1946) (nine new isolates and ATCC no. 6452 = N. R. Smith no. 674 = CN 2199).

The nutritional requirements of these strains were rather more heterogeneous than in most of the other species we studied (Table 5). Furthermore,

Table 5. *Nutritional requirements in* Bacillus pasteurii

(Basal medium adjusted to pH 8·5, incubation at 28°.)

Strain	Number of strains	Nutrients required				
		Ammonia	Amino-acids	Aneurin	Biotin	Nicotinic acid
CN 2199* (=ATCC no. 6452); CN 2715, 2804, 2805	4	+	+	+	−	+
CN 2716, 2717	2	+	+	+	+	−
CN 2713, 2803	2	−	+	+	−	+
CN 2710, 2712	2	−	+	+	+	−

* CN 2199. Clear-cut results as to the requirement or otherwise for biotin in presence of nicotinic acid were difficult to obtain with this strain (see text, p. 533).

some strains had a nutrient requirement satisfied by nicotinic acid. Apart from the aberrant strain CN 2932 of *B. brevis* (see p. 530) none of our strains of mesophilic species in the genus *Bacillus* had a nutritional requirement which could be satisfied by nicotinic acid, i.e. all appeared able to satisfy their nicotinic acid requirements by synthesis. The only other reports of nicotinic acid as a nutrient for an organism of the genus *Bacillus* are those of Cleverdon *et al.* (1949*a*) who had one strain (eurithermophilic, 37–55°) of *B. coagulans* which required nicotinic acid, and the stenothermophilic (55–65°) strains of *Bacillus* reported by the same authors (1949*b*).

The ten strains of *B. pasteurii* examined grew best at 28° and at pH 8·5 in the casein basal medium supplemented by various essential metabolites. Ammonia basal medium did not replace the casein basal medium; thus an

inability to synthesize at least certain amino-acids was shown by all the ten strains.

Earlier workers (Miquel, 1889, 1898; Beijerinck, 1901; quoted by Gibson, 1934*b*, 1935*a*) found that *B. pasteurii* strains did not grow on ordinary laboratory media, e.g. peptone broth, without added urea, and it became generally accepted that the most important criterion for the recognition of *B. pasteurii* strains was inability to grow on 'ordinary' media (presumably at *c.* pH 7) without added urea. Viehoever (1913), however, doubted the need for urea and grew strains of the organism in media simpler than those, for example, used by Beijerinck. Viehoever described growth in an ammonium carbonate mineral medium with glucose and asparagine added. Gibson (1934*b*, 1935*b*) showed that the supposed requirement of urea as a nutrient for many strains of *B. pasteurii* was not absolute; the urea could be replaced by ammonium ion at alkaline pH.

Six of ten strains required ammonium ion in addition to the amino-acids of the casein hydrolysate and certain essential metabolites. Ammonium chloride, to give a final concentration of 1 % (w/v), was added to the basal medium plus essential metabolites, which was adjusted to pH 8·5. Omission of this supplement of 1 % ammonium chloride prevented growth of six of ten strains; this was repeatedly confirmed. We have not compared these two sets of strains for ability to deaminate amino-acids. The existence of a requirement for ammonia, side by side with a requirement for at least certain preformed amino-acids, and an inability to use ammonia as sole N source, as shown by these six ammonia-requiring strains, is striking.

The essential metabolites which were found to be involved in the nutrition of these ten strains of *B. pasteurii* were aneurin, biotin and nicotinic acid. Omission of aneurin from the medium prevented the growth of all ten strains. Omission of biotin prevented the growth of four strains only, the other six strains presumably being able to synthesize this compound. Omission of nicotinic acid prevented the growth only of the six strains which did not require biotin. There was thus a correlation between the biotin and nicotinic acid requirements in that those strains which required biotin did not require nicotinic acid and vice versa. There was no correlation with the requirement for ammonia. Thus these ten strains were either biotin synthesizers or nicotinic acid synthesizers, if the interpretation be accepted that a nutrient requirement for one of the common essential metabolites is due to a defect in the biosynthesis or that essential metabolite. All strains grew better when the four components were present, namely, ammonium ion, aneurin, biotin and nicotinic acid.

Because of the great potency of biotin, clear-cut results with it were never easy to obtain, and usually involved one or more subcultures in biotin-free media, and therefore the possible selection of any biotin-synthesizers in the inocula used. With most species the results were usually unequivocal but with some of the strains of *B. pasteurii*, e.g. CN 2199, the role of biotin was less easy to determine clearly. Usually the generalization that either biotin or nicotinic acid was required as a nutrient was clearly demonstrable but with a few

strains, notably CN 2199, it is possible that both biotin and nicotinic acid are required. The nutritional requirements of *B. pasteurii* suggest problems which require further study.

DISCUSSION

We have isolated from soil specimens of as many of the mesophilic species of the genus *Bacillus* as possible, and tried to name them from the descriptions of Smith *et al.* (1946). Of 296 strains studied 245 clearly belonged to a named species and thirty-two were clearly intermediates between two species; eight remain unidentified and it is possible that further study will clarify their relationship to a described species. Eleven new isolates are believed to represent a previously undescribed species (cf. Proom & Knight, 1950). The classification of Smith *et al.* has, therefore, been submitted to a severe test, and the results are a striking confirmation of the validity of their classification.

Although aberrant strains and intermediate types will always be encountered, comparative work will tend to better the description of the pattern species and consequently to decrease the number of newly isolated strains which would otherwise be classified as intermediate types. For example, we have shown that the inclusion of the egg-yolk reaction and ability to grow anaerobically among the definitive characters of *B. megatherium* and *B. cereus* greatly diminished the number of strains which would otherwise have been classified as intermediates.

Again, growth under strictly anaerobic conditions was useful as a diagnostic character within the genus particularly in connexion with *B. cereus*, *B. licheniformis*, *B. coagulans* and *B. brevis*.

Reaction with egg-yolk also has diagnostic uses apart from its importance in distinguishing between *B. cereus* and *B. megatherium*, where the effect is due to a lecithinase C produced by *cereus* and not by *megatherium*. In contradistinction to the observations of McGaughey & Chu (1948) we found that a number of *Bacillus* species give a reaction with egg-yolk, but one which is restricted to the medium immediately below the growth on egg-yolk agar plates. This restricted reaction helps to distinguish *B. subtilis* from *B. pumilus* and *B. circulans* from other members of morphological group 2.

The survey of nutritional requirements of some 200 strains revealed on the whole a rather unexpected degree of uniformity of nutritional pattern within groups of strains. Also, named cultures and other laboratory strains in general had the same nutritional requirements as newly isolated strains of the same species. That is, the conditions of laboratory cultivation had not led to the selection of nutritional variants. Moreover, specific nutritional patterns appear to be closely related to other specific characters, and to be highly characteristic of species. In some species (e.g. *B. megatherium* and *B. cereus*) all strains, and in other species all but a few strains, had typical nutritional requirements. Exceptional strains were so infrequent that the nutritional pattern could be used as a guide at least in a preliminary allocation of new strains to their species (see Table 6). Thus organisms able to use ammonia only (without growth

Table 6. *Nutritional requirements found for the strains examined in the genus* Bacillus

(The sequence of species follows that of the classification in Bergey's *Manual* (1948), except in group 2, where *brevis* has been put before *alvei*, and *circulans* after these, to emphasize increasing complexity of nutritional requirements in that group. The results for *anthracis* (Gladstone and Brewer *et al.* are included for comparison.)

Organism	Number of strains	Nutrient used: Ammonia	Amino-acids	Aneurin	Biotin	Nicotinic acid
Morphological group 1:						
subtilis	26	+	−	−	−	−
	1	−	+	−	−	−
subtilis var. *aterrimus*	2	+	−	−	−	−
subtilis var. *niger*	1	+	−	−	−	−
licheniformis	12	+	−	−	−	−
pumilus	19	+	−	−	+	−
	2	−	+	−	+	−
subtilis/pumilus	2	+	−	−	−	−
intermediates	2	+	−	−	+	−
coagulans	16	−	+	+	+	−
megatherium	11	+	−	−	−	−
cereus	13	−	+	−	−	−
cereus var. *mycoides*	10	−	+	−	−	−
megatherium/cereus	2	+	−	−	−	−
intermediates	4	−	+	−	−	−
anthracis	.	−	+	+	−	−
Morphological group 2:						
polymyxa	13	+	−	−	+	−
macerans	5	+	−	+	+	−
brevis	9	−	+	−	−	−
	1	−	+	+	+	+
alvei	4	−	+	+	−	−
	1	−	+	+	+	−
circulans	12	−	+	+	+	−
	6	.		Unidentified		.
circulans/alvei	3	−	+	+	+	−
intermediates	3	.		Unidentified		.
Morphological group 3:						
sphaericus	6	−	+	+	−	−
	3	−	+	+	+	−
sphaericus var. *fusiformis*	10	−	+	+	+	−
pasteurii	4	+	+	+	−	+
	2	+	+	+	+	−
	2	−	+	+	+	−
	2	−	+	+	−	+

factors) are likely to be *B. megatherium*, *B. subtilis* (with varieties *aterrimus* and *niger*), or *B. licheniformis*.

Organisms able to use casein-hydrolysate alone or amino-acid mixtures but not ammonia alone, are likely to be *B. cereus* (with var. *mycoides*) or *B. brevis*.

Organisms able to grow with ammonia + biotin but not with ammonia alone are likely to be *B. polymyxa* or *B. pumilus*.

Organisms able to grow with ammonia + biotin + aneurin, but not without one or other of these growth factors are likely to be *B. macerans*.

Organisms unable to use ammonia + aneurin but able to use casein-hydrolysate + aneurin are likely to be *B. anthracis*, *B. alvei* or *B. sphaericus* (the latter not quite so certainly); an additional need for biotin is likely to indicate *B. coagulans*, *B. sphaericus* var. *fusiformis*, less common strains of *B. sphaericus*, or *B. circulans*.

Organisms requiring casein-hydrolysate at pH 8·5, aneurin, ± ammonia, and nicotinic acid or biotin, are likely to be *B. pasteurii*.

In a few cases, already mentioned in the text, the finding of an apparently anomalous nutritional requirement led to a re-examination of what proved to be incorrectly named strains.

In connexion with the exhibition of typical nutritional patterns by groups of newly isolated strains, we do not think that the new isolates were inadvertently selected for given sets of nutritional requirements during isolation. The differential media and conditions of growth used in isolating the strains were not likely to have been selective in this sense. The media used were all nutritionally rich and complex, and therefore unlikely to have favoured the selection of good synthesizers of essential metabolites.

An interesting point seen in Table 6 is that most of the species with the more complex nutritional requirements are in morphological group 3 of Smith *et al.* Whereas all species in groups 1 and 2 so far examined were nutritionally satisfied by ammonia, amino-acids, aneurin and biotin, and often by only one or two of these components, the requirements for the group 3 species were considerably more complex; though *B. firmus* and *B. lentus* of group 1 and *B. laterosporus* and *B. larvae* of group 2 are likely to prove nutritionally complex. No group 3 species can utilize ammonia alone, and all require preformed amino-acids and at least two essential metabolites as growth factors. Furthermore, nicotinic acid was a requirement for more than half of the strains of *B. pasteurii* examined. Apart from a single strain of *B. coagulans* (Cleverdon *et al.* 1949*a*) and an aberrant strain of *B. brevis*, nicotinic acid is not a required nutrient for any mesophilic species of the genus except *B. pasteurii*. The requirement of nicotinic acid by groups of stenothermophilic strains of *Bacillus* observed by Cleverdon *et al.* (1949*b*) has already been mentioned.

What is probably a new species in morphological group 3 of the genus *Bacillus* (Proom & Knight, 1950) also has equally complex nutrient requirements, eleven new isolates requiring amino-acids, aneurin, biotin and pantothenic acid. At present these strains, which represent this proposed new species, are the only ones in the genus known to require pantothenic acid.

We acknowledge with sincere thanks the painstaking and reliable technical assistance of Mr E. Harris and Miss G. M. Trim; without their work this survey would have been impossible. We wish to thank the following donors of strains used here, namely, Dr Ruth E. Gordon, Prof. S. D. Rubbo, Dr E. Schreiner, Dr W. E. van Heyningen, and the Microbiological Research Department (Ministry of Supply), Porton.

Table 7. *The differentiating characters of the species of the genus* Bacillus

The table gives the physiological and typical* nutritional characters of the strains examined in the present work. Bergey's *Manual* (1948), with the insertion of *B. licheniformis* at the appropriate place. *B. anthracis*, *B. laterosporu* completeness.

Morphological group 1 = sporangium not swollen, or only slightly; thin-walled
Morphological group 2 = sporangium swollen; thick-walled oval spore.
Morphological group 3 = sporangium swollen; thick-walled spherical spore.

Species	Growth on				Production of acid (inorganic basal medium)			Voges-Proskauer reaction	Hydrolysis of			Nitrate reduced to nitrite	Urease formation	Citrate utilization	Anaerobic growth	Gibson & Abdel-Malek test	Egg-yolk reaction
	Nutrient agar, pH 7·5	Nutrient agar, pH 6·0	Potato	4% NaCl broth	Glucose	Arabinose	Xylose		Starch	Casein	Gelatin						
Morphological group 1																	
B. subtilis	+++	+	+	+	+	+	+	+	+	+	+	+	−	⊕	−	−	−
B. licheniformis	+++	+	+	+	+	+	+	+	+	+	+	+	−	⊕	+	+	−
B. pumilus	++	+	+	+	+	+	+	+	−	+	+	−	−	+	−	−	tr.
B. coagulans	+	+	±	+	+	⊕	⊕	+	+	−	−	−	−	−	+	−	−
B. firmus	±	−	−	+	−	−	−	−	+	+	+	+	−	−	−	−	−
B. lentus	±	−	−	+	−	−	−	−	−	−	−	−	+	−	−	−	tr.
B. megatherium	+++	+	+	+	+	⊕†	⊕†	−	+	+	+	⊖	var.	+	−	−	−
B. cereus	+++	+	+	+	+	−	−	+	+	+	+	⊕	⊖	⊕	+	−	+
B. anthracis	+++	+	+	+	+	−	−	+	+	+	+	⊕	−	+	+	−	+
Morphological group 2																	
B. polymyxa	+++	+	+	+	+*G*	+*G*	+*G*	+	+	+	+	+	−	−	+	+	var.
B. macerans	±	+	+	+	+*G*	+*G*	+*G*	−	+	−	+	var.	−	−	+	±	tr. *R*
B. circulans	±	+	+	+	+	+	+	−	+	⊕	⊕	var.	⊖	⊖	+	−	−
B. alvei	±	+	+	+	+	−	−	+	+	+	+	−	−	−	+	−	+
B. laterosporus	±	+	+	+	+	+	+	−	−	+	+	+	−	−	+	−	+
B. brevis	±	+	+	⊕	⊕	−	−	−	−	+	+	var.	−	⊕	−	−	tr.
B. larvae	−	.	−	+	.	+	.	.	−	.	+	+	.	.	.	.	.
Morphological group 3																	
B. sphaericus	+	+	±	+	−	−	−	−	−	−	⊕	−	−	−	−	−	−
var. *rotans*	±	−	−	−	−	−	−	−	−	−	⊕	−	−	−	−	−	−
var. *fusiformis*	+	+	±	+	−	−	−	−	−	−	⊕	−	+	−	−	−	−
var. *loehnisii*	±	−	−	+	−	−	−	−	−	−	⊕	−	+	−	−	−	−
B. pasteurii	−	−	−	+	−	−	−	−	−	−	⊕	+	+	−	tr.	−	−

In column 2 (growth on nutrient agar at pH 7·5) the amount of growth is recorded as follows: + + + = abundant g growth; ± = thin growth; ± = poor growth; − = no growth.

In the other columns the following symbols are used: + = positive reaction; ⊕ = usually positive; tr. = faint r reaction; var. = variable; *R* = egg-yolk reaction of 'restricted' type; *G* = gas formation.

* Typical nutritional pattern with respect to nitrogen source and essential metabolites. For detailed analysis of

† Both or one of these sugars fermented.

REFERENCES

Beijerinck, M. W. (1901). *Zbl. Bakt.* (2. Abt.), 7, 33; quoted by Gibson (1934*b*).

Bergey (1948). *Manual of determinative bacteriology*, 6th ed. Edited by Breed, R. S., Murray, E. G. D. & Hitchens, A. P. London: Baillière, Tindall and Cox.

Blackwood, A. C., Neish, A. C., Brown, W. E. & Ledingham, G. A. (1947). Production and properties of 2,3-butanediol. XVII. Fermentation of glucose by strains of *B. subtilis*. *Canad. J. Res.* B, **25**, 56.

Brewer, C. R., McCullough, W. G., Mills, R. C., Roessler, W. G., Herbst, E. J. & Howe, A. F. (1946). Studies on the nutritional requirements of *Bacillus anthracis*. *Arch. Biochem.* **10**, 65.

Burdon, K. L., Stokes, J. C. & Kimbrough, C. E. (1942). Studies of the common aerobic spore-forming bacilli. I. Staining for fat with Sudan black B—Safranine. *J. Bact.* **43**, 717.

Burnside, C. E. (1932). A newly discovered brood disease. *Amer. Bee J.* **72**, 438.

Burnside, C. E. & Foster, R. E. (1935). Studies on the bacteria associated with para-foul-brood. *J. econ. Ent.* **28**, 578.

Christensen, W. B. (1946). Urea decomposition as a means of differentiating *Proteus* and para-colon cultures from each other and from *Salmonella* and *Shigella* types. *J. Bact.* **52**, 461.

Cleverdon, R. C., Pelczar, Jr., M. J. & Doetsch, R. N. (1949*a*). Vitamin requirements of *Bacillus coagulans*. *J. Bact.* **58**, 113.

Cleverdon, R. C., Pelczar, Jr., M. J. & Doetsch, R. N. (1949*b*). The vitamin requirements of stenothermophilic aerobic sporogenous bacilli. *J. Bact.* **58**, 523.

Francis, A. E. & Rippon, J. E. (1949). *Bacillus polymyxa* and its bacteriophages. *J. gen. Microbiol.* **3**, 425.

Gibson, T. (1934*a*). An investigation of the *Bacillus pasteurii* group. I. Description of strains isolated from soils and manures. *J. Bact.* **28**, 295.

Gibson, T. (1934*b*). An investigation of the *Bacillus pasteurii* group. II. Special physiology of the organisms. *J. Bact.* **28**, 313.

Gibson, T. (1935*a*). An investigation of the *Bacillus pasteurii* group. III. Systematic relationships of the group. *J. Bact.* **29**, 491.

Gibson, T. (1935*b*). The urea-decomposing flora of soils. I. Description and classification of the organisms. *Zbl. Bakt.* (2. Abt.), **92**, 364.

Gibson, T. (1944). A study of *Bacillus subtilis* and related organisms. *J. Dairy Res.* **13**, 248.

Gibson, T. & Abdel-Malek, Y. (1945). The formation of carbon-dioxide by lactic acid bacteria and *Bacillus licheniformis* and a cultural method of detecting the process. *J. Dairy Res.* **14**, 35.

Gladstone, G. P. (1939). Inter-relationships between amino-acids in the nutrition of *B. anthracis*. *Brit. J. exp. Path.* **20**, 189.

Hartman, T. L. (1940). The use of Sudan black B as a bacterial fat stain. *Stain Tech.* **15**, 23.

Katznelson, H. (1944). The differentiation of *Bacillus polymyxa* and *Bacillus macerans* on the basis of their vitamin requirements. *J. Bact.* **48**, 495.

Katznelson, H. (1947). Substitution of thiamine by certain amino-acids in the nutrition of *Bacillus para-alvei*. *J. biol. Chem.* **167**, 615.

Katznelson, H. & Lochhead, A. G. (1944). Studies with *Bacillus polymyxa*. III. Nutritional requirements. *Canad. J. Res.* C, **22**, 273.

Katznelson, H. & Lochhead, A. G. (1947). Nutritional requirements of *Bacillus alvei* and *Bacillus para-alvei*. *J. Bact.* **53**, 83.

Katznelson, H. & Lochhead, A. G. (1948). The nutritional requirements of *Bacillus larvae*. *J. Bact.* **55**, 763.

Knight, B. C. J. G. & Fildes, P. (1936). A filter of Pyrex glass and stainless steel for use with Seitz filter-pads. *Biochem. J.* **30**, 1138.

Ledingham, G. A., Adams, G. A. & Stanier, R. Y. (1945). Fermentation of wheat mashes by *Aerobacillus polymyxa*. *Canad. J. Res.* F, **23**, 48.

Lochhead, A. G. (1942). Growth-factor requirements for *Bacillus larvae*, White. *J. Bact.* **44**, 185.

Macfarlane, R. G., Oakley, C. L. & Anderson, C. G. (1941). Haemolysis and the production of opalescence in serum and lecitho-vitellin by the α-toxin of *Clostridium welchii*. *J. Path. Bact.* **52**, 99.

McGaughey, C. A. & Chu, H. P. (1948). The egg-yolk reaction of aerobic sporing bacilli. *J. gen. Microbiol.* **2**, 334.

Miquel, P. (1889, 1898). Quoted in Gibson (1934*b*).

Northrop, J. H., Ashe, L. A. & Senior, J. K. (1919). The biochemistry of *Bacillus acetoethylicum* with reference to the formation of acetone. *J. biol. Chem.* **39**, 1.

Porter, R., McClesky, C. S. & Levine, M. (1937). The fermentative sporulating bacteria producing gas from lactose. *J. Bact.* **33**, 163.

Proom, H. & Knight, B. C. J. G. (1950). *Bacillus pantothenticus* n.sp. *J. gen. Microbiol.* **4**, 539.

Smith, N. R. & Clark, F. E. (1937). A proposed grouping of the mesophilic, aerobic, spore-forming bacilli. *Soil Sci. Soc. Amer. Proc.* **2**, 252.

Smith, N. R., Gordon, R. E. & Clark, F. E. (1946). Aerobic mesophilic spore-forming bacteria. *Misc. Publ. U.S. Dep. Agric.* no. 559. Washington, D.C.

Viehoever, A. (1913). Botanische Untersuchung harnstoffspaltender Bakterien mit besonderer Berücksichtigung der speziesdiagnostisch verwertbaren Merkmale und des Vermögens der Harnstoffspaltung. *Zbl. Bakt.* (2. Abt.), **39**, 209.

(*Received* 8 *March* 1950)

Bacillus pantothenticus (n.sp.)

By H. PROOM and B. C. J. G. KNIGHT

The Wellcome Research Laboratories, Beckenham, Kent

SUMMARY: A new mesophilic species of the genus *Bacillus* was isolated from soil. Within the genus it most nearly resembles *B. circulans* but is clearly distinct in that it produces no motile colonies and no gas from glucose in the presence of an inorganic nitrogen source, hydrolyses casein, liquefies inspissated serum and gives a restricted egg-yolk reaction. It also differs from all other *Bacillus* spp. tested in that its growth is stimulated by 4% NaCl, and that pantothenic acid satisfies a nutritional requirement. The name *Bacillus pantothenticus* is proposed.

During the isolation from soil of mesophilic species of the genus *Bacillus* (Knight & Proom, 1950) an apparently new organism was repeatedly obtained by incubating soil suspensions in 4% NaCl broth, followed by plating on nutrient agar and incubation at 37°. This organism is considered to be a new species; the following description is based on a study of eleven strains isolated from eleven different specimens of soil from widely separated localities in southern England. The methods and media used were similar to those previously described (Smith, Gordon & Clark, 1946; Knight & Proom, 1950).

CHARACTERS

Morphology. Vegetative rods of average size $0{\cdot}5 \times 4{\cdot}0\,\mu.$, slightly curved, usually occurring singly or in bundles consisting of a few organisms. The rods are motile. After 24 hr. growth the organisms are frankly Gram-positive; after further incubation the Gram-staining reaction is variable. The sporangium is swollen and the organism almost drumstick in appearance, the spore being thin-walled, nearly spherical or sometimes spherical. In wet preparations the spores appear spherical more often than in stained preparations. Average spore size in Gram-stained preparations, $1{\cdot}0\,\mu. \times 1{\cdot}0$ to $1{\cdot}5\,\mu.$

Nutrient agar. After 48 hr. at 37° colonies greyish white, opaque with granular surface and irregular edge, 1·5–2·0 mm. diameter.

Nutrient agar slope. After 24 hr. at 37° moderate slightly moist greyish white growth.

Nutrient agar slope (pH 6·0). Growth.

Glucose nitrate agar. No growth.

Semi-solid glucose agar + iron. Gas.

Potato. No growth.

Loeffler blood serum. After 24 hr. at 37° moderate growth with some digestion; marked liquefaction within 7 days.

Nutrient broth. After 24 hr. at 37° faint uniform turbidity with slight ropy deposit. Growth markedly stimulated and improved by addition of 4% NaCl; will give good growth in nutrient broth containing 10% NaCl.

Metabolism. Aerobe, facultative anaerobe. Growth at 37° better than at 28° and usually slightly better than at 45°.

Fermentation tests. Neither acid nor gas from glucose, arabinose or xylose with inorganic basal medium. Acid but no gas from glucose, arabinose and xylose with organic basal medium.

Voges-Proskauer reaction. Negative.

Nitrate reduction to nitrite. Variable.

Urease. Negative.

Catalase. Positive.

Starch hydrolysis. Positive.

Casein hydrolysis. Positive.

Gelatin hydrolysis. Weak positive, the plate-test being positive in 4–7 days; some liquefaction of gelatin-stab within 20 days at 37°.

Egg-yolk reaction. Positive, restricted (Knight & Proom, 1950).

Nutrition. Will grow in a medium containing salts, acid-hydrolysed casein (Knight & Proom, 1950) with aneurin, biotin and pantothenic acid, but not when any one of these three essential metabolites is omitted. Growth is stimulated by the addition of 4 % NaCl. Nine of eleven strains required the intact pantothenic acid molecule, and two strains grew in the presence of β-alanine instead of pantothenic acid.

DISCUSSION

Smith *et al.* (1946) divide the mesophilic species of the genus *Bacillus* into three morphological groups. The new species produces a nearly spherical or spherical spore which appreciably swells the sporangium and therefore belongs to morphological group 3 (see Pl. 1, figs. 3, 4). Comparison of the new species with the named species of the genus *Bacillus* shows that apart from the morphological differences it is more closely related to *B. circulans* than to any other species in the genus. Besides the morphological differences the new species can be distinguished from *B. circulans* by the following characters.

Cultures on nutrient agar after 24 hr. at 37° are frankly Gram-positive and the growth on nutrient agar after 48 hr. is moderate, greyish white and slightly moist. The colonial appearance on nutrient agar is characteristic and motile colonies have not been observed (see Pl. 1, figs. 1, 2). The growth after 24 hr. (37°) in nutrient broth is faint with a slight ropy deposit. This growth is stimulated and improved by the addition of 4 % NaCl and the species will grow well in nutrient broth containing 10 % NaCl. The growth of *B. circulans* in broth is not stimulated by the addition of 4 % NaCl and although an occasional strain of *B. circulans* grows in nutrient broth containing 6 % NaCl and one of our strains grew in nutrient broth containing 8 % NaCl, none of the strains we have examined grew in nutrient broth containing 10 % NaCl.

The new species does not produce acid from glucose with basal medium containing only inorganic nitrogen. The new species, in contradistinction to *B. circulans*, hydrolyses casein, liquefies inspissated serum and gives a positive restricted egg-yolk reaction.

The nutritional requirements of the new species are different from those of *B. circulans*, and from any of the other species of this genus which we have examined (Knight & Proom, 1950), in that the initial growth is stimulated by

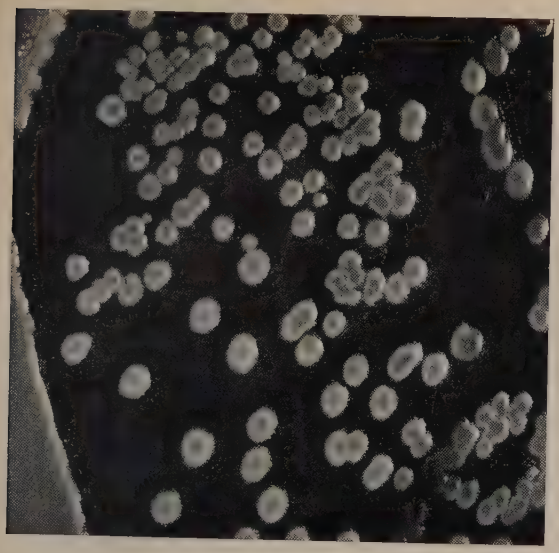


Fig. 1

Fig. 3

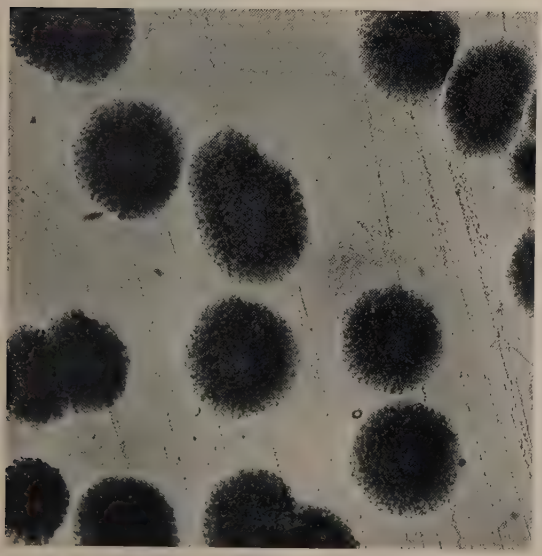


Fig. 2

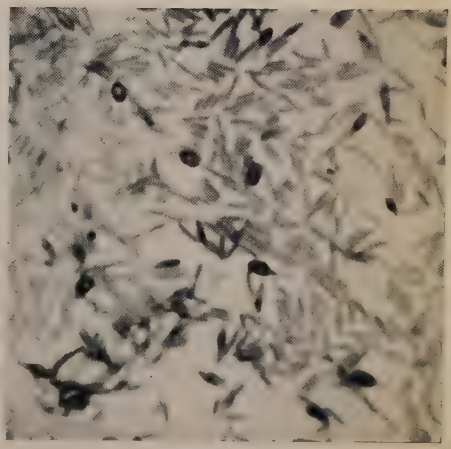


Fig. 4

H. Proom & B. C. J. G. Knight—*Bacillus pantothenticus* (n.sp.). Plate 1

the addition of 4 % NaCl and that pantothenic acid satisfies a nutritional requirement, the intact pantothenic acid molecule being required for most strains.

We propose *Bacillus pantothenticus* as the name of this new species, derived from pantothenic acid which is a characteristic nutrient for this organism. Six strains of *B. pantothenticus* have been deposited at the National Collection of Type Cultures, of which one (CN 3028) is the type strain, now N.C.T.C. no. 8162.

We are indebted to Mr E. Harris for valuable technical assistance and to Mr E. A. Jones for the photographs.

REFERENCES

Knight, B. C. J. G. & Proom, H. (1950). A comparative survey of the nutrition and physiology of mesophilic species in the genus *Bacillus*. *J. gen. Microbiol.* 4, 508.

Smith, N. R., Gordon, R. E. & Clark, F. E. (1946). Aerobic mesophilic spore-forming bacteria. *Misc. Publ. U.S. Dep. Agric.* no. 559. Washington, D.C.

EXPLANATION OF PLATE

Fig. 1. Colonies of *B. pantothenticus*, strain CN 3028, on nutrient agar after 48 hr. at 37°. Seen by reflected light. Magnification ×2.

Fig. 2. Colonies of *B. pantothenticus*, strain CN 3028, on nutrient agar after 48 hr. at 37°. Seen by transmitted light. Magnification ×8.

Fig. 3. Photomicrograph of Gram-stained film of *B. pantothenticus*, strain CN 3028. Magnification ×2000.

Fig. 4. Photomicrograph of Gram-stained film of *B. circulans*. Magnification ×2000.

(*Received* 28 *March* 1950)

[*The Editors of the* Journal of General Microbiology *accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from authors.*]

THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its Ninth General Meeting at Nottingham University on Thursday and Friday, 29 and 30 September 1949. The following communications were made:

COMMUNICATIONS

Studies on the Correlation of the Physical and Biological Properties of *Haemophilus pertussis*. By J. Ungar, A. M. James and W. K. Stevens (*Greenford*)

In previous communications the authors have reported some biological differences between virulent and avirulent strains of *H. pertussis*, particularly their growth requirements and precipitability by aluminium phosphate. The physical and biological properties of some twenty-five strains (some recently isolated and some maintained under laboratory conditions for several years) were redetermined because of some recent findings. It would seem from the latest experiments that certain degrees of virulence to mice after intranasal infection are correlated with the ability of the strains to produce capsules on suitable media after 24 hr. incubation. At the same time they are able to grow in a chemically partly defined medium only if starch is present, whereas avirulent strains grow in absence of starch. The agglutinin titre of different strains gives less contrasting results. Virulent strains are completely precipitated by aluminium phosphate and dissolved in presence of bile salts. Virulent strains are most lethal to mice (by intranasal infection) when 48 hr. old. They also show a comparatively higher haemagglutinating ability than avirulent strains.

Aspects of the Trace-element Nutrition of *Streptomyces griseus*. By C. G. C. Chesters and G. N. Rolinson (*Nottingham*)

Experiments were carried out to determine the relationship between trace elements and the production of antibiotic substances by the organism, and also to investigate whether those trace elements already shown to be required by higher plants, algae, fungi and bacteria are also required by actinomycetes. The medium was rendered free from trace elements by extraction with chloroform solutions of dithizone and oxine.

Zinc, iron and copper were found to be indispensable to *S. griseus*, and the minimum amounts required for optimum growth were 0·5, 0·2 and 0·05 p.p.m. respectively. Concentrations of these metals up to 50 p.p.m. had little effect on growth except in the case of iron when growth was slightly depressed.

The curves for antibiotic production were parallel to those for growth in the

case of zinc and copper with the same minimum requirements for optimum activity. Concentrations up to 50 p.p.m. of copper had no significant effect on antibiotic production but similar concentrations of zinc depressed the titre to less than 50 % normal. In the case of iron, although 0·2 p.p.m. were sufficient for maximum growth, antibiotic production was very low at this concentration. 1 p.p.m. of iron was the minimum amount required for maximum antibiotic production. 50 p.p.m. of iron depressed the titre to less than 50 % of the maximum level.

No deficiency in growth or antibiotic production could be obtained on a manganese-deficient medium. However, on this same medium *Aspergillus niger* displayed marked manganese deficiency. Using this organism, the quantity of inoculum of *Streptomyces griseus* used in the experiments was found to contain less than 0·005 p.p.m. of manganese, and it could only be assumed that if manganese is required by *S. griseus* this need is satisfied by an extremely small quantity.

The Nutrition of *Clostridium tetanomorphum*. By K. A. Sims and D. D. Woods (*Oxford*)

This organism has been described as growing with glutamate as main carbon, nitrogen and energy source (Barker, 1939); this was further investigated in view of the probability (Gale, 1947) that energy is needed for the entry of glutamate into the cell of Gram-positive organisms.

Cl. tetanomorphum is now found to grow on a purified casein hydrolysate supplemented with glucose, salts, pantothenate, Tween 80 and *p*-aminobenzoate *or* pteroyl-glutamate. The last-named is consistently 2–4 fold more active than *p*-aminobenzoate. The organism is almost insensitive to sulphonamide in the presence of pteroyl-glutamate. Growth, slightly delayed, occurs when glucose is omitted provided that glutamate concentration is increased. Good growth is also obtained when the hydrolysate is replaced by high concentrations (10^{-2}M) of L-glutamate plus cystein, aspartate, and tryptophan. Omission of glucose again leads to slower growth: DL-glutamate was almost ineffective in the above experiments; the reason for this, and also the role of Tween 80, is under investigation.

REFERENCES

Barker, H. A. (1939). *Arch. Mikrobiol.* **10**, 376.
Gale, E. F. (1947). *J. gen. Microbiol.* **1**, 53.

The Release of Cellular Constituents from *Staphylococcus aureus* treated with Cetyltrimethylammonium Bromide. By M. R. J. Salton and A. E. Alexander (*Cambridge*)

It has been shown by Hotchkiss (1944) that when bacterial cells were treated with certain surface active compounds in bactericidal concentrations, there was a release of nitrogenous and phosphorus-containing substances into the

suspending fluid. The initial amount of N and P released was close to the amount extractable by trichloracetic acid. Gale & Taylor (1947) have found that free amino-acids in the internal environment of *Streptococcus faecalis* leak into the suspending medium when tryocidin, cetyltrimethylammonium bromide or Aerosol O.T. or phenol are present.

To test the possibility that CTAB may also release ultra-violet absorbing constituents from cells, 16 hr. broth cultures of *Staph. aureus* were harvested, washed three times with distilled water, suspended in distilled water and treated as follows: one-half of the suspension was added to distilled water, the other half was added to a solution of CTAB of final concentration 90 μg./ml. The final concentration of bacteria was approx. 1·5 mg. dry weight/ml. Under these conditions most of the detergent is adsorbed by the cells and 99·99 % of the organisms killed within 10 min. Samples from the two suspensions held at room temperature (*c.* 20° C.) were taken at various periods, the cells centifuged off and the supernatant cleared by further centrifugation.

Using the Beckman spectrophotometer the ultra-violet spectra of supernatants from untreated and CTAB-treated cells were found to have a maximum absorption at a wave-length of 260 mμ and a marked increase in the 260 mμ absorption of the supernatant from CTAB-treated cells was observed.

The rate of release of 260 mμ absorbing material and inorganic phosphorus at 0, 20 and 30° C. was determined and a parallel relationship between the release of the two constituents was apparent at the three temperatures. The rate of release was found to be dependent on temperature.

That the parallel relationship between the appearance of 260 mμ absorbing material and inorganic P in the suspending fluid, under experimental conditions reported here, represents the course of the release of cellular constituents and not enzymatic breakdown of the cells, is supported by the following evidence. The appearance of 260 mμ absorbing material and inorganic P in the suspending fluid was followed concurrently with the release of glutamic acid from *Staph. aureus* treated with 90 μg./ml. CTAB at 20° C. The glutamic acid content of the supernatant was estimated by the decarboxylase method of Gale (1947). The results showed that a parallel relationship exists for the course of the release of all three cellular constituents into the suspending fluid. Further, the total amounts of the three constituents in the suspending fluid from CTAB-treated cells did not differ markedly from the amounts released from suspensions of cells held in a boiling water-bath for 10 min.

The estimation of 260 mμ absorbing material in the supernatant from detergent-treated cells would therefore appear to be a sensitive and rapid method for determining alterations in the permeability of the cell membrane. This method has been used in studying the effect of CTAB concentration on the disorganization of the cell membrane of *Staph. aureus* at 20° C. The rate of release of 260 mμ absorbing material was found to be linearly proportional to the concentration of CTAB over the range 45–135 μg. CTAB/ml., above which a maximum rate of release is achieved. With 22·5 μg. CTAB/ml. the rate of release of 260 mμ absorbing material is only twice that of the control.

A preliminary examination of the supernatant for purines and pyrimidines

has been made, using the partition paper chromatography method of Markham & Smith (1949). This has revealed the presence of a number of components absorbing in the ultra-violet, two of which have been found to be free adenine and uracil.

The possibility of using the measurement of 260 mμ absorbing material as an indication of cell membrane damage to Gram-negative bacteria treated with detergents is suggested from a few preliminary observations with *Escherichia coli* H.

REFERENCES

Gale, E. F. (1947). *J. gen. Microbiol.* **1**, 53.
Gale, E. F. & Taylor, E. S. (1947). *J. gen. Microbiol.* **1**, 77.
Hotchkiss, R. D. (1944). *Advanc. Enzymol.* **4**, 153.
Markham, R. & Smith, J. D. (1949). *Nature, Lond.*, **163**, 250.

The Bacterial Surface: Effect of Cetyltrimethylammonium Bromide on the Electrophoretic mobility. By Kenneth McQuillen (*Cambridge*)

A study has been made of the changes in the surface charge of *Esch. coli*, *Strep. faecalis*, and strains of *Staph. aureus*, in phosphate buffer = 0·01, pH = 7·0, in the presence of the cationic detergent cetyltrimethylammonium bromide (CTAB) 1–250 M. The charge of *Esch. coli* falls progressively as the CTAB concentration is increased, being neutralized at about 200 M and becoming reversed at higher concentrations. This confirms the findings of Dyar & Ordal (1946), working with cetylpyridinium chloride. However, in contradistinction to their finding that the many different species of bacteria studied all exhibit an approximately similar pattern of behaviour, it is now found that the Streptococci and Staphylococci show a sharp discontinuity. After an initial small decrease in charge there is an abrupt rise between CTAB concentrations of 50 and 100 M, followed by a rapid fall, these organisms bearing only a small positive or negative charge in the presence of 250 M-CTAB. Data concerning the adsorption by *Staph. aureus* of CTAB using the method of estimation of Salton & Alexander (1949), is also presented and it is found that saturation occurs at the same concentration as the maximum in the mobility/CTAB concentration curves. Other findings at variance with the earlier work are that the charge in a given concentration of CTAB varies with time and also depends on the suspension density of cells.

The Formation of Ethyl Acetate by Yeast. By J. L. Peel (*Cambridge*)

Cultures of *Hansenula anomala*, when grown aerobically on media containing glucose, produce ethyl acetate. The amount of ester produced is too large to be accounted for by the reversal of a simple esterase reaction.

A rapid micro method for ester determination has been developed based on the conversion of the ester to the corresponding hydroxamic acid. This method has been applied to the study of ester formation from acetate plus ethanol by washed suspensions of *H. anomala*. The process is essentially aerobic and highest

ester yields were obtained at pH 4·6, at a temperature of 25°, using yeast harvested from 48 hr. cultures.

The ester formed from acetate plus ethanol has been provisionally identified as ethyl acetate.

The effect of 10^{-5}M-di-isopropylfluorophosphonate indicates the presence of two distinct systems, one synthesizing and the other breaking down the ester.

The Assimilation of Glutamic Acid Derivatives by *Staphylococcus aureus*. By E. F. Gale (*Cambridge*)

Glutamic acid will pass into the staphylococcal cell only if glucose is being fermented; this may mean that a metabolite of glutamic acid is formed which passes through the osmotic barrier of the cell wall and is reconverted to glutamic acid within the cell. Substances, other than glutamic acid, which will give rise to free internal glutamic acid when incubated with the cell in the presence of glucose are: glutamine, α-L-glutamyl-L-glutamic acid and glutathione. Glutamine appears to enter the cell more rapidly, and the dipeptide slightly less rapidly, than glutamic acid itself. There is no evidence that the dipeptide is hydrolysed prior to passing through the cell wall. The temperature coefficients for the transfer are lower for glutamine and glutamyl-glutamic acid than for glutamic acid itself.

Two substances have been found which will enter the cell and give rise to free internal glutamic acid in the absence of glucose: N-phosphoryl-glutamic acid and diethyl-glutamic-ester. N-phosphoryl-glutamic acid enters the cell at a rate *c.* 70% that of the entry of glutamic acid with glucose, and the temperature coefficient for the process is intermediate between that obtained for the transfer of glutamic acid and of the dipeptide. Diethyl glutamic acid appears to diffuse through the cell wall and glutamic acid is liberated within the cell by esterase. The rate of glutamic acid appearance is limited by the rate of esterase action, but the process has a temperature coefficient lower than that of the other derivatives studied. The passage into the cell of all derivatives, other than the ester, is inhibited by treatment with penicillin.

Derivatives which do not give rise to internal glutamic acid in the presence or absence of glucose are: pyrrolidone carboxylic acid, α-amino-butyric acid, N-acetyl-glutamic acid, pteroylglutamic acid, pteroyltriglutamic acid, the *p*-aminobenzoic acid-polyglutamate complex of yeast extracts.

Further Observations on the Group or Species Specific Ninhydrin Positive Substances produced by Bacteria. By A. J. Woiwod and H. Proom (*Beckenham*)

In a previous paper (Proom & Woiwod, 1949) the authors described the appearance of a number of ninhydrin-positive spots on papergrams of filtrates of bacterial strains grown on a casein hydrolysate medium. These spots were

not present in the basal medium. Since in most cases they did not correspond in position to any of the amino-acids present in the media it was tentatively suggested that they might be polypeptide in nature. The ninhydrin-positive substances produced by *Serratia marcescens*, *Shigella paradysenteriae*, *Proteus vulgaris* and *Clostridium tetani* have been eluted from papergrams and further studied. The substance produced by *Ser. marcescens* resists acid hydrolysis, gives a positive Sakaguchi reaction and matches arginine on two-dimensional chromatography. The substance produced by *Sh. paradysenteriae* at acid pH is γ-amino butyric acid produced by specific decarboxylation of glutamic acid. The substances produced by *Pr. vulgaris* are polypeptides containing a high proportion of amino-acids in the valine and leucine groups and are similar in R_F values and gross amino-acid composition to those produced by *Cl. tetani*.

REFERENCE

Proom, H. & Woiwod, A. J. (1949). *J. gen. Microbiol.* 3, 319.

Do *Fusiformis necrophorus* and *Streptobacillus moniliformis* show a Primitive Form of Sexuality according to Mellon's views? By E. Klieneberger-Nobel (*London*)

When *Fusiformis necrophorus* and *Streptobacillus moniliformis* are subcultured at regular short intervals on suitable media they develop into cultures which show at first only small regularly shaped bacillary forms with chromatinic structures of a normal arrangement. After some hours of growth they form small elements containing one chromatinic granule only and not showing a cell wall. These elements coalesce if they are in close contact. Two or more elements can coalesce and grow out into the peculiar L bodies which have been described by myself and other authors. Much later or when newly subcultured these bodies may reproduce normal bacilli.

Pure Culture Isolation of *Nitrosomonas*. By Jane Meiklejohn (*Harpenden*)

Nitrosomonas europaea (Winogradsky), an autotrophic bacterium which oxidizes ammonia to nitrate, has been obtained in pure culture from Rothamsted soil. The method used was a modification of Winogradsky's method involving the following steps: (1) building up the numbers of bacteria in an enrichment culture; (2) transfers on an improved liquid medium; (3) removal of the cells from the chalk debris in a culture with carbon dioxide; (4) making poured plates on silica gel, and picking colonies into liquid medium.

A Tentative Method of obtaining a 'Balance Sheet' of the Soil Micro-flora. By C. G. C. Chesters and A. Apinis (*Nottingham*)

In an attempt to obtain a balance sheet of the fungal population of soils, three methods of isolation have been applied simultaneously to each soil sample. Special immersion tubes, allowing access through capillary orifices to sterile

medium inside the tube, are inserted in the soil by removing a core of soil. This core is treated in a washing apparatus to separate the organic matter in different grades. Isolations from the organic matter and from the soil suspension left by the washing give qualitative estimates of the mycelium living in the debris and of the spore content of the total soil respectively. The results from the immersion tubes provide evidence of mycelium growing in the soil *in situ*. The results from all three methods are combined to give a balance sheet for the several layers of the soil profile.

Measurements of Rate of Mutation of Flagellar Antigenic Phase in *Salmonella typhi-murium*. By B. A. D. Stocker (*London*)

When melted sloppy agar containing antibody for one phase is layered over *Salm. typhi-murium* colonies, swarming organisms form wide diffuse opaque zones round colonies of non-agglutinated phase, and narrow sharp-edged zones round colonies of agglutinated phase. The increase in proportion of cells of 'mutant' phase during prolonged logarithmic growth of single-cell cultures has been measured; the mutation rate is calculated from the initial (linear) rate of increase. Rates of mutation from group to specific varied from 10^{-4} to $4{\cdot}7 \times 10^{-3}$ per generation cycle, and rates of the reverse mutation from 10^{-5} to $8{\cdot}6 \times 10^{-4}$. In all nine strains investigated (including two *binns* strains) the rate of mutation from group to specific was greater than the rate of the reverse mutation, so that the strains tend towards an equilibrium composition in which the specific phase predominates: this equilibrium was demonstrated in one rapidly mutating strain.

Some Observations on the Classification of the Genus *Bacillus*. By H. Proom (*Beckenham*)

Smith, Gordon & Clark's (1946) classification of the Gram-positive aerobic mesophilic spore-forming bacteria has been followed in the identification of isolates from several hundred specimens of soil. One of the objectives of this study is the more adequate definition of the species in order to decrease the number of types isolated which would otherwise be described as intermediates. This is illustrated by additions, complementing those given by Smith *et al.* to the descriptions of the following species. These amended descriptions considerably reduce the number of intermediate types recognized. *B. megatherium*—strictly aerobic, does not produce lecithinase, will grow in the absence of growth factors and with ammonia as the only source of nitrogen. *B. cerus*—will grow anaerobically, produces lecithinase and will grow in the absence of growth factors but requires amino-acids as a source of nitrogen. *B. subtilis*—strictly aerobic, will grow in the absence of growth factors and with ammonia as the only source of nitrogen. *B. licheniformis*—will grow anaerobically, anaerobically ferments glucose to give a high concentration of glycerol. *B. pumilus*—strictly aerobic, will grow in a medium containing ammonia as the only source of

nitrogen, but requires biotin as a growth factor. The growth requirements of these organisms were determined during a nutritional study of the genus, of which a preliminary report has been given (Knight & Proom, 1949).

REFERENCES

Smith, N. R., Gordon, R. E. & Clark, F. E. (1946). *Misc. Publ. U.S. Dep. Agric.* no. 559.

Knight, B. C. J. G. & Proom, H. (1949). *J. gen. Microbiol.* 3, *P.* xxv.

The Mechanical Transmission and some Properties of Potato Paracrinkle virus. By F. C. Bawden, B. Kassanis and H. L. Nixon (*Harpenden*)

Observations on the Cultivation of *Haemophilus pertussis*. By Betty Dawson, Enid Farnworth, D. E. Nicholson and J. W. McLeod (*Leeds*)

The various elements in Bordet-Gengou medium which promote the growth of *recently isolated strains* of *H. pertussis* have been investigated.

Blood is essential but especially the serum component. Starch and glycerol do not seem to be important. The nitrogenous elements in the potato extract appear to be the more valuable.

All peptones investigated inhibit growth slightly or markedly in 1% concentration.

If peptone is excluded meat extract is usually a better base for Bordet than potato extract.

Of eighteen amino-acids, serine and tryptophane appeared to be inhibitory and glutamic and aspartic acids favourable to growth. The remainder were indifferent in so far as investigated. Aspartic acid has the additional advantage of inhibiting *H. influenzae*. Thirty per cent blood, meat extract agar + 0·05% aspartic acid is well worth trial as a medium for primary isolation of *H. pertussis*.

(Several text-books depart from the original formula in recommending inclusion of peptone or tryptic digest in Bordet-Gengou medium.)

Non-specific Lysis observed in the Bacteriophage typing of *Staphylococcus aureus*. By Joan E. Rippon (*London*)

In the routine bacteriophage typing of staphylococci a number of strains were encountered which gave plaques with practically all the typing phages; the areas of lysis, however, did not resemble those ordinarily observed, and when neat phages were used only a few typical areas of lysis appeared.

With these strains, but with no others, plaques could often be induced by dropping saline on to the plates. By picking such plaques to a small quantity of broth, emulsifying and spinning, the lytic agent could be obtained in the supernatant. Dilutions of these supernatants dropped on to agar plates spread

with the original strain, and with the routine phage-propagating strains, gave evidence of phage action against the original strain and one or more of a common group of the propagating strains.

It was concluded that some staphylococci carry a phage which in certain circumstances can lyse the strain which carries it, and that such phages from different strains are closely related to one another.

Osmophilic Yeasts from Concentrated Orange Juice. By M. Ingram (*Cambridge*)

Concentrated orange juice is fermented by yeasts. They can grow in solutions of sugar-concentration up to 70 %, and of pH 3 or even more acid; the optimum sugar solution is dextrose about 30 %, and the optimum pH about 4·5. Few of the isolated strains are obligately osmophilic. Only dextrose, laevulose and mannose are fermented: it is noteworthy that sucrose, abundant in the juice, is not. The optimum temperature varies from 25° to 35° C. with increasing sugar-concentration in the medium. Such yeasts can be isolated on media with 50 % dextrose + 1 % citric acid + 1 % peptone, on which little else grows. Transferred annually on this medium and kept at 0° C. for several years, they preserve their osmophilic characters; in ordinary media they do not. In high-sugar media, they produce capsular material in which the cells remain embedded in groups. Especially on potato some of them sporulate, after conjugation. They show a strong tendency to change from smooth to rough colony form on solid media of low sugar-content, or from round to long cells in liquid media—these changes seem to be associated with staling in the medium. In general, they resemble *Zygosaccharomyces* species already described from honey, etc.

DEMONSTRATIONS

H. Proom and A. J. Woiwod. (*Beckenham*) 'The examination by paper chromatography of some aspects of the nitrogen metabolism of bacteria.'

C. B. Taylor. (*Welwyn*) 'An improved colony illuminator.'

C. B. Taylor. (*Welwyn*) 'Silical gells prepared by an easy base-exchange method.'

C. G. C. Chesters and G. N. Rolinson. (*Nottingham*) 'Trace element nutrition of fungi.'

C. G. C. Chesters and A. Apinis. (*Nottingham*) 'Methods of isolating fungi from soil.'

C. L. Hannay. (*Reading*) 'A new counting chamber.'

M. Lumb. (*Nottingham*) 'Antibiotic-producing organisms.'

L. Dickinson. (*Nottingham*) 'Laboratory techniques with animal viruses.'

[*The Editors of the* Journal of General Microbiology *accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from authors.*]

THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its Tenth General Meeting in the Royal Institution, London, on Tuesday and Wednesday, 4 *and* 5 *April* 1950. *The following communications were made:*

COMMUNICATIONS

The Decarboxylation of Succinic Acid by Washed Suspensions of Rumen Bacteria. By S. R. Elsden and A. Kaars Sypesteyn (*Sheffield*)

Sypesteyn (1948, 1949) reported the isolation of an anaerobic, Gram-positive, streptococcus from the rumen of the ox which fermented cellulose with the production of succinic and acetic acids. A similar organism has now been isolated from the rumen of sheep (Sypesteyn, unpublished work). The strain from the sheep ferments both cellulose and cellobiose, and succinic and acetic acids are major end-products. Whilst this organism is always present in the rumen only small amounts of succinic acid are found in the rumen liquor. On the other hand, large amounts of propionic acid are produced by rumen bacteria under both *in vivo* and *in vitro* conditions (Elsden, 1945; Marston, 1948). The demonstration by Johns (1949) of the decarboxylation of succinic acid to propionic acid and CO_2 suggested an explanation of the absence of succinic acid from the rumen.

A technique for the preparation of active suspensions of rumen bacteria has been developed, and these suspensions decompose succinic acid quantitatively to propionic acid and CO_2. Johns (personal communication) has made similar observations on washed suspensions of rumen bacteria prepared from New Zealand sheep. Succinic added to the rumen *in vivo* is converted to propionic acid. The use of washed suspensions of rumen bacteria in the study of ruminant digestion is discussed.

REFERENCES

Elsden, S. R. (1945). *J. exp. Biol.* **22**, 51.
Johns, A. T. (1949). *Nature, Lond.*, **164**, 620.
Marston, H. R. (1948). *Biochem. J.* **42**, 564.
Sypesteyn, A. K. (1948). Thesis, Leyden University.
Sypesteyn, A. K. (1949). *Antonie van Leeuwenhoek*, **15**, 49.

The Reduction of Nitrate by Rumen Bacteria. By Dyfed Lewis (*Sheffield*)

Feeding ruminants on diets containing nitrate results in a methaemoglobinaemia which may prove fatal. It is believed that the nitrate is reduced to nitrite by the rumen bacteria and that the nitrite so formed produces the methaemoglobinaemia.

$NaNO_3$ (25 g.) introduced directly into the rumen of a sheep 15 hr. after the last meal disappeared within 10 hr. with the formation of both nitrite and ammonia, and the conversion of 60 % of the blood haemoglobin to the methaemoglobin. Nitrite added to the rumen was partly reduced to ammonia and partly absorbed into the blood-stream with the formation of methaemoglobin.

The nature of the hydrogen donators in this reaction have been investigated using washed suspensions of rumen bacteria. Hydrogen was the most active and nitrate, nitrite and hydroxylamine were rapidly reduced to ammonia; the pH optima of these reactions were pH 6·2, 5·6 and 6·2–6·5 respectively. Succinate, formate, glucose and lactate were also active, but the volatile acids acetate, propionate and butyrate were inactive.

A Possible New Species in the Genus *Bacillus*. By H. Proom and B. C. J. G. Knight (*Beckenham*)

Further Comparative Study of Nutritional Requirements in the Genus *Bacillus*. By B. C. J. G. Knight and H. Proom (*Beckenham*)

A survey of the nutrition of mesophilic species in the genus *Bacillus* has been continued. New isolates and laboratory strains of all except the rarer species have now been examined. On the whole a surprising degree of uniformity of specific nutritional pattern has been found, so that it is possible to make a preliminary allocation of new isolates to their probable species by nutritional screening. In summary, the species were satisfied by the combinations of nutrients shown below, with very few exceptional strains.

B. subtilis, *B. licheniformis* and *B. megatherium* grew with ammonia; *B. cereus*, *B. cereus* var. *mycoides* and *B. brevis* with amino-acids, not ammonia alone; *B. pumilus* and *B. polymyxa* grew with ammonia + biotin, *B. macerans* with ammonia + biotin + aneurin.

The following species all grew with amino-acids (not with ammonia only) and the essential metabolites indicated: *B. alvei*, aneurin; *B. circulans* and *B. coagulans*, aneurin + biotin; *B. sphaericus*, aneurin or aneurin + biotin; *B. sphaericus* var. *fusiformis*, aneurin + biotin.

The strains of *B. pasteurii* showed most heterogeneity. All needed amino-acids + aneurin, some needed NH_4, in addition, and all needed either biotin or nicotinic acid depending on the strain, i.e. those which required biotin synthesized nicotinic acid and vice versa.

A probably new species required amino-acids, aneurin, biotin and pantothenic acid.

Resistance to Infection with *Salmonella enteritidis* and *Mycobacterium tuberculosis* of Mice Fed Different Diets. By J. W. Howie (*Bucksburn*)

1. Six experimental diets of natural food for mice were tested for their capacity to support reproduction, growth and resistance to infection.

2. There were clear-cut differences in the relative efficiency of the diets for reproduction and growth. Differences in the relative efficiency of the diets

for resistance to infection with tubercle bacilli or salmonella organisms were less marked and consistent, and the best diet for reproduction (diet 3) was the poorest for resistance to infection.

3. The dietary effects upon resistance to infection were quite clear in some tests but less marked in others. The relatively low order of the differences between diets, and the inconsistency of the results between different tests renders it necessary to continue inquiry into the relations between diet and resistance to infection.

4. When 6 % of the whole wheat in the B diet of Sherman was replaced by 5 % casein and 1 % calcium carbonate, the capacity of the diet to support reproduction and growth of mice was seriously impaired.

The Production of Non-Capsulated Avirulent Variants by *Bacillus anthracis* and its Implication on Taxonomy. By H. P. Chu (*Cambridge*)

The production of non-capsulated avirulent variant by *Bacillus anthracis* was first discovered by Sterne (1937). He found that on 50 % serum agar in air containing 65 % CO_2 *B. anthracis* produced, instead of the rough medusa-head colony, a markedly smooth mucoid growth of capsulated cells which then gave rise to a rough outgrowth. Subculture from the outgrowth gave a variant which differed from the parent culture in its inability to produce capsules and mucoid growth under these special conditions and in its loss of pathogenicity.

In the course of our comparative study of *B. anthracis* and the saprophytic aerobic sporing bacilli, this important dissociation was thoroughly investigated. Our attention was directed to the implication of the dissociation on bacterial taxonomy. Experiments will be described to show that the avirulent variant was present in nearly all the laboratory cultures of *B. anthracis* examined and also in nature.

The finding that *B. anthracis* can give rise with such readiness to the non-capsulated avirulent variant has opened up a difficult taxonomical problem. According to the existing classification, *B. anthracis* is defined as an aerobic sporing bacillus capable of causing anthrax in animals. We have now an organism which is a direct descendant of *B. anthracis*, but as a loss of its pathogenicity is more likely to be classified as *B. cereus* than as *B. anthracis*. This shows that *B. anthracis* and *B. cereus* are closely related, and that the criteria used in the present classification for their separation are inadequate. More extensive investigation is therefore needed to see if other differences between them can be found. In this paper certain differences between *B. anthracis* and *B. cereus* in their physiological and serological properties will be presented, and the recent suggestion of Smith, Gordon & Clark (1946) that *B. anthracis* should be regarded as a pathogenic variety of *B. cereus* and renamed as '*B. cereus* var. *anthracis*' will be discussed.

REFERENCES

Sterne, M. (1937). *Onderstepoort J. vet. Sci.* 8, 271.

Smith, N. R., Gordon, R. E. & Clark, F. E. (1946). *Misc. Publ. U.S. Dep. Agric.* no. 559.

The Deamidation of Nicotinamide by *Lactobacillus arabinosus* 17–5. By D. E. Hughes and D. H. Williamson (*Sheffield*)

Washed suspensions of *Lactobacillus arabinosus* 17–5 were found to synthesize cozymase when nicotinic acid or nicotinamide was added together with glucose (18–24 mμmol./mg. dry wt./hr.). The amounts of nicotinic acid removed from the medium and the amounts of cozymase synthesized were the same. Added nicotinamide (50 mμmol./mg. dry wt.) disappeared from the medium much more rapidly ($>$600 mμmol./mg. dry wt./hr.) than nicotinic acid, but this was due to its conversion into nicotinic acid and ammonia. Glucose is not required for the deamidation of nicotinamide. In the absence of glucose equimolar amounts of nicotinic acid and ammonia are formed. The deamidation also occurs in cell-free extracts. The activity of these was destroyed by boiling. Glutamine, asparagine, ornithine, arginine, urea, cozymase and nicotinamide nucleotide did not yield ammonia with the preparations which can thus be used as a specific reagent for the estimation of nicotinamide.

Lb. bulgaricus and *Lb. helveticus* (ϵ) deamidated nicotinamide at the same rate as *Lb. arabinosus*. No deamidation occurred with two strains of *Staphylococcus aureus* and *Streptococcus haemolyticus*, or with *Bacterium aerogenes*, *Proteus vulgaris* and *Streptococcus faecalis*.

The Formation and Germination of Bacterial Spores Studied by Phase Contrast and Slide-Cell Culture. By R. J. V. Pulvertaft (*London*)

Bacteria can readily be grown on cover-slips covered with nutrient agar as 'Slide-Cell Cultures'. When examined on a warm stage by phase contrast morphological changes can be observed in individual bacteria in relation to their growth phases, and to changes in their environment.

By cutting away one-half of the agar surface, and running solutions of test material under the cover-slip, the action of these materials on growing organisms may be investigated. Examples are the neutralization of penicillin by penicillinase, and the effect of vitamin B_{12} on organisms growing on a B_{12} deficient medium.

Agar and distilled water provide a mechanism on which a great many organisms will grow. Their translucency under these conditions make them admirable subjects for phase-contrast study, and many spore-forming species can be studied in relation to spore formation and spore germination. Such preparations are also well adapted for cinemicrography.

Cytological Studies on Tissue Cultures Infected with Fowl-Plague Virus. By C. E. Challice and T. H. Flewett (*London*)

Attempts were made to investigate the intracellular growth of fowl-plague virus in tissue culture.

Lying-drop tissue cultures were made on 'Formvar' membranes supported on cover-slips. The explants were of amnion and of pectoral muscle from

10-day-old chick embryos. The medium (normal amniotic fluid from fowl-plague infected eggs) was added to the cultures, which were examined by the phase-contrast microscope after varying periods of incubation.

Intranuclear inclusions appeared in fibroblasts 24 hr. after inoculation and in epithelium after 48 hr. The nucleolus first increased in size and then became granular and highly refractile, finally disintegrating into fragments.

The cultures were then fixed and mounted on grids for electron microscopic examination. Long filaments, mainly arranged in bundles, were first found in fibroblasts at 36 hr., and in epithelium 48 hr., after inoculation. Some filaments showed segmentation into small round bodies. It is considered that these observations suggest the existence of a growth cycle of fowl-plague virus.

The Serology of *Bacillus polymyxa*. By Sheila N. Davies (*Beckenham*)

The spore, somatic and flagellar antigens of *Bacillus polymyxa* are serologically distinct.

The spore antigen is common to the thirty-nine strains of *B. polymyxa* tested, and appears to be species specific; no cross reactions have been observed with spore suspensions of other species of the genus *Bacillus*.

The somatic antigen is complex. Absorption tests failed to show any clear-cut groups, and minor cross-reactions occur with O suspensions of other species.

The flagellar antigen is strain specific on first isolation, but after maintaining the strains on nutrient agar for some months, variation occurs, with the appearance of group antigens. One of the group antigens is common to nearly all strains of *B. polymyxa* and also to the seven strains of *B. macerans* tested. Cross-reactions have also occurred with isolated strains of other species.

The antigens of *B. polymyxa* are complex, but the spore antigen appears to be the simplest and most likely to be of use in classification.

The Measurement of the Aeration of Biological Culture Media. By W. S. Wise (*Epsom*)

The aeration of submerged cultures can be assessed by measuring the rate of solution of oxygen into the medium and the rate of consumption of oxygen by the organism. The latter rate can be determined by measuring with a polarograph the concentration of dissolved oxygen as it is consumed from a culture medium initially saturated with air. The rate of solution can be estimated by aerating an oxygen-free medium and measuring the concentration of dissolved oxygen at suitable intervals.

The maximum titre reached in streptomycin fermentations depended on the rate of solution of oxygen. No specific effect of vessel-size or agitation was noticed. The titre increased with aeration until the rate of solution was equal to the peak oxygen demand of the cultures. The titre then became independent of the rate of solution of oxygen as is expected theoretically.

The Two Opposing Effects of Subtilin on Bacteria. By T. M. Asher (*London*)

The antibiotic subtilin has two general effects upon susceptible organisms, these being lysis and inhibition. The degree of respiratory inhibition and the loss of viability are directly proportional to the concentration of subtilin. However, lysis is indirectly proportional to subtilin concentration up to a certain point (e.g. when the ratio of μg. subtilin per organism is 1×10^{-9}) where it then becomes directly proportional. It is therefore suggested that organisms are susceptible to the lytic ability of the antibiotic only at a particular phase in the life cycle and, if inhibited before reaching that phase, will not lyse.

The cells need not be actively growing to lyse, but the amount of lysis of a culture would be quite small in that case since only a small percentage of the cells are to be found in the lytic susceptible phase at any one instant. Lysis of a large proportion of cells in a culture depends upon the cells being able to grow until this particular phase is reached.

An application of this knowledge to the field of therapeutics seems necessary, particularly in the case of subtilin where large doses of the antibiotic have proved to be excessively toxic.

The Control of Pyrogen Formation During the Refining of Antitoxic Sera. By A. E. Francis (*Beckenham*)

Bacterial Macromolecules. I. The Isolation of Deoxyribonucleic Acid from Virulent and Avirulent Strains of *Haemophilus pertussis*. By W. G. Overend, M. Stacey, M. Webb (*Birmingham*) and J. Ungar (*Greenford*)

Haemophilus pertussis cells contain a relatively large amount of deoxyribonucleic acid which may be extracted from the organisms with 2 % sodium cholate. A method is reported for the isolation of deoxyribonucleic acid from the mechanically disintegrated pertussis organisms. The material can be obtained in apparently undegraded form and free from ribonucleic acid and proteins. It does contain a polysaccharide component, and in the virulent strain this appears to be in firm combination with the nucleic acid. The yield of deoxyribonucleic acid is greater from the avirulent than from the virulent strains of the organism. Some properties of the nucleic acids will be mentioned. The relevance of this work in providing further evidence of the differences in biological properties of virulent and avirulent strains such as agglutinability by type specific serum, precipitability by aluminium phosphate and solubility in bile and sodium hydroxide, is discussed.

Induced Mutation of *Bacillus anthracis*. By J. Tomcsik (*Basel*)

A moderately virulent strain of *Bacillus anthracis* was subcultured in a broth containing different extracts of a mucoid strain of *B. mesentericus*. The growth was plated on ordinary agar. In 15 % of the subcultures smooth colonies of *B. anthracis* appeared, composed of well-capsulated *B. anthracis* with moderate

virulence. No motility was observed. The same changes could be induced when the original strain was inoculated in mice together with a large amount of *B. mesentericus*. The smooth colonies of *B. anthracis* were transferred in series on agar medium. After several months the type of the colonies was essentially the same; however, the capsule formation appeared only after 24–48 hr. and was preceded by a motile phase. The morphology of the bacilli was identical with that of the initial strain. The virulence disappeared. The capsular polypeptide was identical with that of *B. anthracis*, but the somatic antigen was different from that of any member of the *Bacillus* group studied so far.

Some Effects of Cobalt on the Growth and Metabolism of *Proteus vulgaris*.

By A. L. Schade and H. B. Levy (*New York*)

Cobaltous ions, in concentrations ranging from 10^{-6} to 10^{-3} M depending upon the composition of the culture medium, can inhibit the growth of a number of species of bacteria, including *Proteus vulgaris* (Schade, 1949). If cobalt is present in the nutrient broth at the time of inoculation, the cells of *P. vulgaris* show no increase in size nor do they divide. When cobalt is added to cultures during the logarithmic phase of growth, cell multiplication is halted in less than one generation. The addition of histidine, in a molar ratio to cobalt of 2 : 1 effectively binds the metal (Hearon, Burk & Schade, 1949) and permits initiation or resumption of growth after an intervening lag period.

Respiratory measurements made in conjunction with the growth studies show that the Q_{O_2} of the growing cells is approximately twice that of cells in their early lag phase in the same medium. The endogenous respiration of *P. vulgaris* in saline or phosphate buffer is affected little, if at all, by growth-inhibiting concentrations of cobalt. The respiration of cells in their early lag phase in nutrient broth, however, is sensitive to similar cobalt concentrations but to a smaller extent than the heightened growth respiration of rapidly dividing cells in which the inhibition may reach 80–90 %. When histidine is added to a culture of rapidly dividing cells whose respiration has been inhibited by cobalt, the respiration rises in about 20 min. to the level prevailing before cobalt addition, followed by a lag period of approximately 1 hr. during which the growth respiratory rate is maintained without concurrent growth of the culture. Following this lag, the cells begin to multiply and the respiratory rate increases proportionately. From these observations it appears that a heightened respiration is associated with, but is not sufficient for, the growth of *P. vulgaris*.

REFERENCES

Schade, A. L. (1949). *J. Bact.* **58**, 811.
Hearon, J. Z., Burk, D. & Schade, A. L. (1949). *J. Nat. Cancer Inst.* **9**, 337.

FILMS

A. Pijper (*Pretoria*). 'The effect of penicillin on the motility of *Bacillus proteus*.'

Westminster Hospital Medical School and Wright-Fleming Institute. 'The motility of *Bacillus proteus* on penicillin agar' (phase contrast).

R. J. V. Pulvertaft (*London*). 'The phenomenon of heat control of movement of *Bacillus proteus*.'

DEMONSTRATIONS

M. R. Pollock (*London*). 'A method of simultaneously testing large numbers of single colonies, from mixed bacterial cultures, for production of penicillinase.'

A. C. Stirling and M. K. Stevens (*Edinburgh*). 'Colony counts on strips of agar in tubes.'

Agricultural Research Council Plant Virus Research Unit (*Cambridge*). (*a*) 'Virus diseases of insects.' (*b*) 'Composition of nucleic acids of some bacteria and viruses.'

C. B. Taylor and W. K. Smith (*Welwyn*). 'Growth of micro-organisms on silica gel media.'

H. P. Chu (*Cambridge*). 'The differentiation of *Bacillus anthracis* from the saprophytic aerobic sporing bacilli.'

C. E. Challice and T. H. Flewett (*London*). 'The technique of cytological studies on tissue cultures infected with fowl-plague virus.'

Index of Authors

Entries marked with an asterisk (*) are those for which only a title is given and of which there are no summaries.
Small roman numerals refer to pages in Proceedings of the Society for General Microbiology.

ALEXANDER, A. E. *see* SALTON, M. R. J. ii
APINIS, A. *see* CHESTERS, C. G. C. vi
ASHER, T. M. The two opposing effects of subtilin on bacteria xvi

BARBOUR, R. G. H. & VINCENT, J. M. The bacteriostatic action of phenol, benzoic acid, and related compounds on *Bacterium aerogenes* 110
BARNES, J. M. *see* PROOM, H. 270
BARTON, A. A. Some aspects of cell division in *Saccharomyces cerevisiae* 84
BASU, S. N. & BOSE, R. G. Factors affecting the fruiting of *Chaetomium* species 132
BAWDEN, F. C., KASSANIS, B. & NIXON, H. L. The mechanical transmission and some properties of potato paracrinkle virus 210
BAWDEN, F. C., KASSANIS, B. & NIXON, H. L. The mechanical transmission and some properties of potato paracrinkle virus viii
BAWDEN, F. C. & PIRIE, N. W. Some factors affecting the activation of virus preparations made from tobacco leaves infected with a tobacco necrosis virus 464
BAWDEN, F. C. & PIRIE, N. W. Some effects of freezing in the leaf, and of citrate *in vitro*, on the infectivity of a tobacco necrosis virus 482
BISSET, K. A. The sporulation of *Clostridium tetani* 1
BISSET, K. A. The differentiation of certain genera of Bacteriaceae by the morphology of the microcyst stage 413
BISSET, K. A. & MOORE, F. W. *Jensenia*, a new genus of the Actinomycetales 280
BOSE, R. G. *see* BASU, S. N. 132
BRODIE, J. & SHEPHERD, W. The effect of the gas-phase on differential inhibition of intestinal bacilli 102

CHALLICE, C. E. & FLEWETT, T. H. Cytological studies on tissue cultures infected with fowl-plague virus xiv
CHESTERS, C. G. C. & APINIS, A. A tentative method of obtaining a 'balance sheet' of the soil micro-flora vi
CHESTERS, C. G. C. & ROLINSON, G. N. Aspects of the trace-element nutrition of *Streptomyces griseus* i
CHU, H. P. The production of non-capsulated avirulent variants by *Bacillus anthracis* and its implication on taxonomy xiii
CROWLEY, N. The degradation of starch by strains of Group A streptococci having related antigens 156
CRUMP, L. M. The influence of the bacterial environment on the excystment of amoebae from soil 16
CUTTS, N. S. & RAINBOW, C. Studies of a yeast exacting towards *p*-aminobenzoic acid 150

DAGLEY, S., DAWES, E. A. & MORRISON, G. A. Factors influencing the early phases of growth of *Aerobacter aerogenes* 437
DAVIES, S. N. The serology of *Bacillus polymyxa* xv
DAWES, E. A. *see* DAGLEY, S. 437
DAWSON, B., FARNWORTH, E., NICHOLSON, D. E. & MCLEOD, J. W. Observations on the cultivation of *Haemophilus pertussis* viii

EDWARD, D. G. FF. An investigation of pleuropneumonia-like organisms isolated from the bovine genital tract 4
EDWARD, D. G. FF. An investigation of the biological properties of organisms of the pleuropneumonia group, with suggestions regarding the identification of strains 311
ELSDEN, S. R. & SYPESTEYN, A. K. The decarboxylation of succinic acid by washed suspensions of rumen bacteria xi
ENDE, M. VAN DEN & TURNER, G. S. Further observations on a filtrable agent isolated from bovine lumpy skin disease 225
ENDE, M. VAN DEN. The utilization of amino-acid solutions by virus-infected eggs, studied by paper chromatography 277
EVANS, D. G. & PROPHET, A. S. Enzymes of *Clostridium welchii* Type A and *Clostridium histolyticum* that disintegrate decalcified human tooth dentine 360

FARNWORTH, E. *see* DAWSON, B. viii
FISHER, P. J. A note on the immediate death-rate [of dried bacterial cultures] 455
FLEMING, A. Further observations on the motility of *Proteus vulgaris* grown on penicillin agar 457
FLEMING, A., VOUREKA, A., KRAMER, I. R. H. & HUGHES, W. H. The morphology and motility of *Proteus vulgaris* and other organisms cultured in the presence of penicillin 257
FLETCHER, L. I. *see* KING, H. K. 238
FLEWETT, T. H. *see* CHALLICE, C. E. xiv
FOLEY, G. E. & SHWACHMAN, H. Some observations on a Streptomycin-dependent strain of *Staphylococcus aureus* 141

FORSYTH, W. G. C. & WEBLEY, D. M. The reducing sugars liberated during the bacterial synthesis of polysaccharides from sucrose 87
*FRANCIS, A. E. The control of pyrogen formation during the refining of antitoxic sera xvi
FULLER, A. T. & HORTON, J. M. Marcescin, an antibiotic substance from *Serratia marcescens* 417

GALE, E. F. The assimilation of glutamic acid derivatives by *Staphyloccoccus aureus* v
GRAY, E. & SMITH, J. D. A new aquatic nitrogen-fixing bacterium from three Cambridgeshire chalk streams 281

HANNAY, C. L. The serological identity of a yellow-pigmented *Streptococcus* 294
HART, P. D'A. & MOSS, B. The distinction of licheniformin from subtilin by cross-reactions with antibiotic-resistant strains of *Mycobacterium phlei* 244
HARVEY, D. G. *see* LOVELL, R. 493
HEATLEY, N. G. A versatile fermentation sampling arrangement 410
HILLS, G. M. Chemical factors in the germination of spore-bearing aerobes: observations on the influence of species, strain and conditions of growth 38
HIRSCH, A. The assay of the antibiotic nisin 70
HOPKINS, R. H. & PENNINGTON, R. J. The effect of growth-factor deficiencies upon fermentation of glucose by yeasts 171
HORTON, J. M. *see* FULLER, A. T. 417
HOWIE, J. W. Resistance to infection with *Salmonella enteritidis* and *Mycobacterium tuberculosis* of mice fed different diets xii
HUGHES, D. E. & WILLIAMSON, D. H. The deamidation of nicotinamide by *Lactobacillus arabinosus* 17–5 xiv
HUGHES, W. H. *see* FLEMING, A. 257
HUTNER, S. H. Anaerobic and aerobic growth of purple bacteria (Athiorhodaceae) in chemically defined media 286

INGRAM, M. Osmophilic yeasts from concentrated orange juice ix

JAMES, A. M. *see* UNGAR, J. i
JAMES, A. M. *see* UNGAR, J. 345
JEBB, W. H. H., KNOX, R. & TOMLINSON, A. H. Some factors influencing the rate of formation of tetrathionase 374

KASSANIS, B. *see* BAWDEN, F. C. viii
KASSANIS, B. *see* BAWDEN, F. C. 210
KING, H. K. & FLETCHER, L. I. The production of γ-aminobutyric acid by *Bacterium coli* Wilson, Type I 238
KING, H. K. & STEIN, J. H. The non-toxicity of *Bacillus anthracis* cell material 48
KLECZKOWSKA, J. A study of phage-resistant mutants of *Rhizobium trifolii* 298
KLECZKOWSKI, A. Interpreting relationships between the concentrations of plant viruses and numbers of local lesions 53
KLECZKOWSKI, A. & NIXON, H. L. An electron-microscope study of potato virus *X* in different states of aggregation 220
KLIENEBERGER-NOBEL, E. Do *Fusiformis necrophorus* and *Streptobacillus moniliformis* show a primitive form of sexuality according to Mellon's views? vi
KNIGHT, B. C. J. G. & PROOM, H. A comparative survey of the nutrition and physiology of mesophilic species in the genus *Bacillus* 508
KNIGHT, B. C. J. G. & PROOM, H. Further comparative study of nutritional requirements in the genus *Bacillus* xii
KNIGHT, B. C. J. G. *see* PROOM, H. 539
*KNIGHT, B. C. J. G. *see* PROOM, H. xii
KNOX, R. Tetrathionase: the differential effect of temperature on growth and adaptation 388
KNOX, R. *see* JEBB, W. H. H. 374
KRAMER, I. R. H. *see* FLEMING, A. 257

LACEY, M. S. The antibiotic properties of fifty-two strains of *Fusarium* 122
LAWLEY, D. N. *see* STIRLING, A. C. 339
LEVY, H. B. *see* SCHADE, A. L. xvii
LEWIS, D. The reduction of nitrate by rumen bacteria xi
LOVELL, R. & HARVEY, D. G. A preliminary study of ammonia production by *Corynebacterium renale* and some other pathogenic bacteria 493

MCLEOD, J. W. *see* DAWSON, B. viii
MCQUILLEN, K. The bacterial surface: effect of cetyltrimethylammonium bromide on electrophoretic mobility iv
MATTHAEI, E. Simplified fluorescence microscopy of tubercle bacilli 393
MEIKLEJOHN, J. The isolation of *Nitrosomonas europaea* in pure culture 185
MEIKLEJOHN, J. Pure culture isolation of *Nitrosomonas* vi
MILES, A. A. *see* MILES, E. M. 22
MILES, E. M. Red-leg in tree-frogs caused by *Bacterium alkaligenes* 434
MILES, E. M. & MILES, A. A. The relation of toxicity and enzyme activity in the lecithinases of *Clostridium bifermentans* and *Clostridium welchii* 22
MITCHELL, P. Spectrophotometric estimation of nucleic acid in bacterial suspensions 399
MOORE, F. W. *see* BISSET, K. A. 280
MOORE, W. T. & TAYLOR, C. B. An improved colony illuminator 448
MORRISON, G. A. *see* DAGLEY, S. 437
MOSS, B. *see* HART, P. D'A. 244
MUGGLETON, P. W. *see* UNGAR, J. 345

NICHOLSON, D. E. *see* DAWSON, B. viii
NIXON, H. L. *see* BAWDEN, F. C. 210
NIXON, H. L. *see* BAWDEN, F. C. viii
NIXON, H. L. *see* KLECZKOWSKI, A. 220

OAKLEY, C. L. & WARRACK, G. H. The alpha, beta and gamma antigens of *Clostridium histolyticum* (Weinberg & Séguin, 1916) 365

OLSEN, E. Obituary notice—S. Orla-Jensen, 1870–1949 107
ORBELL, W. G. *see* PROOM, H. 270
OVEREND, W. G., STACEY, M., WEBB, M. & UNGAR, J. Bacterial macromolecules. I. The isolation of deoxyribonucleic acid from virulent and avirulent strains of *Haemophilus pertussis* xvi

PEEL, J. L. The formation of ethyl acetate by yeast iv
PEGLER, H. F. *see* UNGAR, J. 345
PENNINGTON, R. J. *see* HOPKINS, R. H. 171
PIRIE, N. W. *see* BAWDEN, F. C. 464, 482
PORTERFIELD, J. S. Classification of the streptococci of subacute bacterial endocarditis 92
POWELL, J. F. Factors affecting the germination of thick suspensions of *Bacillus subtilis* spores in L-alanine solution 330
PRINGSHEIM, E. G. The bacterial genus *Lineola* 198
PROOM, H. Some observations on the classification of the genus *Bacillus* vii
PROOM, H. & KNIGHT, B. C. J. G. *Bacillus pantothenticus* (n.sp.) 539
*PROOM, H. & KNIGHT, B. C. J. G. A possible new species in the genus *Bacillus* xii
PROOM, H., WOIWOD, A. J., BARNES, J. M. & ORBELL, W. G. A growth-inhibitory effect on *Shigella dysenteriae* which occurs with some batches of nutrient agar and is associated with the production of peroxide 270
PROOM, H. *see* KNIGHT, B. C. J. G. xii
PROOM, H. *see* KNIGHT, B. C. J. G. 508
PROOM, H. *see* WOIWOD, A. J. v
PROOM, H. *see* WOIWOD, A. J. 501
PROPHET, A. S. *see* EVANS, D. G. 360
PULVERTAFT, R. J. V. The formation and germination of bacterial spores studied by phase contrast and slide-cell culture xiv

RAINBOW, C. *see* CUTTS, N. S. 150
RHODES, M. Viability of dried bacterial cultures 450
RIPPON, J. E. Non-specific lysis observed in the bacteriophage typing of *Staphylococcus aureus* viii
ROBINOW, C. F. A note on stalked forms of viruses 242
ROLINSON, G. N. *see* CHESTERS, C. G. C. i

SALTON, M. R. J. & ALEXANDER, A. E. The release of cellular constituents from *Staphylococcus aureus* treated with cetyltrimethylammonium bromide ii
SHEPHERD, W. *see* BRODIE, J. 102
SCHADE, A. L. & LEVY, H. B. Some effects of cobalt on the growth and metabolism of *Proteus vulgaris* xvii
SHWACHMAN, H. *see* FOLEY, G. E. 141
SIMS, K. A. & WOODS, D. D. Nutrition of *Clostridium tetanomorphum* ii
SMITH, J. D. *see* GRAY, E. 281
STACEY, M. *see* OVEREND, W. G. xvi
STEIN, J. H. *see* KING, H. K. 48
STEVENS, M. K. *see* STIRLING, A. C. 339
STEVENS, W. K. *see* UNGAR, J. i
STIRLING, A. C., STEVENS, M. K. & LAWLEY, D. N. Colony counts on strips of agar in tubes 339
STOCKER, B. A. D. Measurements of rate of mutation of flagellar antigenic phase in *Salmonella typhi-murium* vii
SYPESTEYN, A. K. *see* ELSDEN, S. R. xi

TAYLOR, C. B. An improved method for the preparation of silica gel media for microbiological purposes 235
TAYLOR, C. B. *see* MOORE, W. T. 448
TOMCSIK, J. Induced mutation of *Bacillus anthracis* xvi
TOMICH, E. G. *see* UNGAR, J. 345
TOMLINSON, A. H. *see* JEBB, W. H. H. 374
TOSIC, J. & WALKER, T. K. *Acetobacter acidum-mucosum* Tosic & Walker, n.sp., an organism forming a starch-like polysaccharide 192
TURNER, G. S. *see* ENDE, M. VAN DEN 225

UNGAR, J., JAMES, A. M., MUGGLETON, P. W., PEGLER, H. F. & TOMICH, E. G. The cultivation of *Haemophilus pertussis* in partially defined liquid media 345
UNGAR, J., JAMES, A. M. & STEVENS, W. K. Studies on the correlation of the physical and biological properties of *Haemophilus pertussis* ix
UNGAR, J. *see* OVEREND, W. G. xvi

VINCENT, J. M. *see* BARBOUR, R. G. H. 110
VOUREKA, A. *see* FLEMING, A. 257

WALKER, T. K. *see* TOSIC, J. 192
WARRACK, G. H. *see* OAKLEY, C. L. 365
WEBB, M. *see* OVEREND, W. G. xvi
WEBLEY, D. M. *see* FORSYTH, W. G. C. 87
WHITE, P. B. A note on the globular forms of *Vibrio cholerae* 36
WILLIAMSON, D. H. *see* HUGHES, D. E. xiv
WISE, W. S. The measurement of the aeration of biological culture media xv
WOIWOD, A. J. & PROOM, H. Further observations on the group or species specific ninhydrin-positive substances produced by bacteria v
WOIWOD, A. J. & PROOM, H. Identification of characteristic extracellular ninhydrin-positive substances produced by some bacteria 501
WOIWOD, A. J. *see* PROOM, H. 270
WOODS, D. D. *see* SIMS, K. A. ii

Index of Subjects

Entries marked with an asterisk (*) are those for which only a title is given and of which there are no summaries.
Small roman numerals refer to pages in Proceedings of the Society for General Microbiology.

Acetobacter acidum-mucosum, new species characterized (Tosic & Walker) 192
Actinomycetales, *Jensenia*, a new genus of (Bisset & Moore) 280
Activation of tobacco necrosis virus preparations and factors affecting (Bawden & Pirie) 464
Adaptive enzyme, tetrathionase, differential effect of temperature on growth and adaptation (Knox) 388
Adaptive enzyme, tetrathionase, factors affecting rate of formation of (Jebb *et al.*) 374
Aeration of biological culture media, measurement of (Wise) xv
Aerobacter aerogenes, factors affecting early phases of growth of (Dagley *et al.*) 437
L-Alanine solutions, germination of *B. subtilis* spores in (Powell) 330
Amino-acid utilization in virus-infected eggs (van den Ende) 277
p-Aminobenzoic acid, nutrient essential for a yeast (Cutts & Rainbow) 150
α-Aminobutyric acid detected in culture filtrates (Woiwod & Proom) 501
γ-Aminobutyric acid detected in culture filtrates (Woiwod & Proom) 501
γ-Aminobutyric acid, produced by *Bact. coli* Wilson Type I (King & Fletcher) 238
δ-Aminopentanoic acid detected in culture filtrates (Woiwod & Proom) 501
Ammonia production by *C. renale* (Lovell & Harvey) 493
Amoebae of soil, effect of bacteria on excystment of (Crump) 16
Amylase in group A streptococci (Crowley) 156
Antibiotic marcescin from *Serratia marcescens* (Fuller & Horton) 417
Antibiotic nisin, assay of (Hirsch) 70
Antibiotics, licheniformin and subtilin, distinguished by cross-reactions with resistant forms of *Myco. phlei* (Hart & Moss) 244
Antibiotics produced by *Fusarium* spp. (Lacey) 122
Antigens α, β and γ, of *Cl. histolyticum* (Oakley & Warrack) 365
Arginase in *Corynebacterium* spp. (Lovell & Harvey) 493
Assay of antibiotic nisin (Hirsch) 70
Athiorhodaceae, anaerobic and aerobic growth in defined media (Hutner) 286

Bacillus anthracis, induced mutation (Tomcsik) xvi
Bacillus anthracis, non-toxicity of cell material (King & Stein) 48
Bacillus anthracis, production of non-capsulated avirulent variants by (Chu) xiii
Bacillus genus, comparative survey of nutrition and physiology of mesophilic species of (Knight & Proom) 508
Bacillus genus, nutritional requirements of (Knight & Proom) xii
Bacillus genus, observations on classification of (Proom) vii
Bacillus pantothenticus (n.sp.) a new mesophilic species of *Bacillus* (Proom & Knight) 539
Bacillus polymyxa, serology of (Davies) xv
Bacillus, species of, chemical factors in spore-germination (Hills) 38
Bacillus subtilis spores, germination, factors affecting (Powell) 330
Bacteria, two opposing effects of subtilin on (Asher) xvi
Bacteriaceae, differentiation of certain genera by morphology of microcyst stage (Bisset) 413
Bacterial spores, formation and germination studied by phase contrast and slide-cell culture (Pulvertaft) xiv
Bacteriophage-typing of *Staph. aureus*, non-specific lysis observed in (Rippon) viii
Bacteriostasis of *Bact. aerogenes*, by phenol, benzoic acid and related compounds (Barbour & Vincent) 110
Bacterium aerogenes, bacteriostasis by phenol, benzoic acid and related compounds (Barbour & Vincent) 110
Bacterium alkaligenes, red-leg in tree-frogs caused by (Miles, E. M.) 434
Bacterium coli, Wilson Type I, γ-aminobutyric acid produced by (King & Fletcher) 238
Bovine Lumpy Skin disease, filtrable agent from, further study (van den Ende & Turner) 225
Budding of *Saccharomyces cerevisiae* (Barton) 84

Cell division in *Saccharomyces cerevisiae* (Barton) 84
Cetyltrimethylammonium bromide, release of cell constituents of *Staph. aureus* treated with (Salton & Alexander) ii
Cetyltrimethylammonium bromide, effect on electrophoretic mobility of bacteria (McQuillen) iv
Chaetomium spp., factors affecting fruiting of (Basu & Bose) 132
Chromatographic study of amino-acid utilization in virus-infected eggs (van den Ende) 277

Chromatography in identification of characteristic ninhydrin-positive extra-cellular substances (Woiwod & Proom) 501
Chromatography, paper, in study of reducing sugars liberated during polysaccharide synthesis (Forsyth & Webley) 87
Chromatography, paper, in study of group and species specificity in production of ninhydrin-positive substances by bacteria (Woiwod & Proom) v
Classification of streptococci in subacute endocarditis (Porterfield) 92
Classification in genus *Bacillus*, observations on (Proom) vii
Clostridium bifermentans, lecithinase of, relation of enzymic and toxic effects of, to *Cl. welchii* lecithinase (Miles & Miles) 22
Clostridium histolyticum, α, β and γ antigens of (Oakley & Warrack) 365
Clostridium histolyticum, enzymes from, disintegrating decalcified human tooth dentine (Evans & Prophet) 360
Clostridium tetani, characteristic polypeptides produced by (Woiwod & Proom) 501
Clostridium tetani, sporulation of (Bisset) 1
Clostridium tetanomorphum, nutrition of (Sims & Woods) ii
Clostridium welchii, lecithinase of, relation to *Cl. bifermentans* lecithinase (Miles & Miles) 22
Clostridium welchii, Type A, enzymes from, disintegrating decalcified human tooth dentine (Evans & Prophet) 360
Cobalt, some effects of, on the growth and metabolism of *P. vulgaris* (Schade & Levy) xvii
Colony counts on agar strips in tubes (Stirling *et al.*) 339
Colony illuminator, an improved (Moore & Taylor) 448
Comparative survey of nutrition and physiology of mesophilic species in genus *Bacillus* (Knight & Proom) 508
Corynebacterium equi, arginase and urease in (Lovell & Harvey) 493
Corynebacterium ovis, arginase and urease in (Lovell & Harvey) 493
Corynebacterium pyogenes, arginase and urease in (Lovell & Harvey) 493
Corynebacterium renale, ammonia production by (Lovell & Harvey) 493
Counting colonies, on agar strips in tubes (Stirling *et al.*) 339
CTAB (*see* Cetyltrimethylammonium bromide)
Cultivation of *H. pertussis* in partially-defined media (Ungar *et al.*) 345
Culture media, biological, measurement of aeration of (Wise) xv

Death-rate, immediate, of dried bacteria (Fisher) 455
Decarboxylation of succinic acid by washed suspensions of rumen bacteria (Elsden & Sypesteyn) xi
Dentine, decalcified human tooth, enzymes disintegrating, from *Cl. welchii* Type A, and *Cl. histolyticum* (Evans & Prophet) 360
Dried bacterial cultures, immediate death-rate of (Fisher) 455
Dried bacterial cultures, viability of (Rhodes) 450

Electron-microscope study of potato virus *X* aggregation (Kleczkowski & Nixon) 220
Electrophoretic mobility of bacteria, effect of CTAB on (McQuillen) iv
Endocarditis, subacute bacterial, classification of streptococci of (Porterfield) 92
Enzyme, adaptive tetrathionase, factors affecting rate of formation of (Jebb *et al.*) 374
Enzyme, tetrathionase, differential effect of temperature on growth and adaptation (Knox) 388
Enzymes disintegrating decalcified dentine, from *Cl. welchii* and *Cl. histolyticum* (Evans & Prophet) 360
Escherichia coli (*see Bacterium coli*)
Ethyl acetate, formation of, by *Hansenula anomala* (Peel) iv
Excystment of soil amoebae, effect of bacteria (Crump) 16

Fermentation of glucose by yeasts, effect of growth-factor deficiencies on (Hopkins & Pennington) 171
Fluorescence microscopy of tubercle bacilli (Matthaei) 393
Fowl-plague virus, cytological studies on tissue cultures infected with (Challice & Flewett) xiv
Fruiting of *Chaetomium* spp., factors affecting (Basu & Bose) 132
Fusarium spp., antibiotics produced by (Lacey) 122
Fusiformis necrophorus, queried primitive form of sexuality in (Klieneberger-Nobel) vi

Germination in spore-bearing aerobes, chemical factors in, influence of species, strain, conditions of growth (Hills) 38
Germination of *B. subtilis* spores in L-alanine solutions (Powell) 330
Glutamic acid derivatives, assimilation by *Staph. aureus* (Gale) v
Growth of *A. aerogenes*, early phases of, factors affecting (Dagley *et al.*) 437
Growth-factor deficiencies, effect on fermentation of glucose by yeasts (Hopkins & Pennington) 171

Haemophilus pertussis, correlation of physical and biological properties of (Ungar, James & Stevens) i
Haemophilus pertussis, cultivation in partially defined media (Ungar *et al.*) 345
Haemophilus pertussis, isolation of deoxyribonucleic acid from virulent and avirulent strains (Overend *et al.*) xvi
Haemophilus pertussis, observations on cultivation of (Dawson *et al.*) viii
Halophilic species, *B. pantothenticus* (Proom & Knight) 539
Hansenula anomala, formation of ethyl-acetate by (Peel) iv

Identification of strains of pleuropneumonia group (Edward) 311
Illuminator for colonies, an improved (Moore & Taylor) 448
Infectivity of tobacco necrosis virus, effects of freezing and citrate on (Bawden & Pirie) 482
Infectivity to plant viruses, relation of concentration to numbers of local lesions (Kleczkowski) 53
Inhibition, differential, of intestinal bacilli, effect of gas-phase on (Brodie & Shepherd) 102
Intestinal bacilli, effect of gas-phase on differential inhibition of (Brodie & Shepherd) 102

Jensenia, a new genus of Actinomycetales (Bisset & Moore) 280
Jensenia canicruria n.sp. (Bisset & Moore) 280

Lactobacillus arabinosus 17–5, deamidation of nicotinamide by (Hughes & Williamson) xiv
Lecithinase of *Cl. bifermentans* and of *Cl. welchii*, relation of (Miles & Miles) 22
Licheniformin and subtilin distinguished by cross-reactions with resistant forms of *Myco. phlei* (Hart & Moss) 244
Lineola, a new bacterial genus (Pringsheim) 198
Lineola articulata n.sp. (Pringsheim) 198
Lineola longa n.sp. (Pringsheim) 198
Lumpy Skin Disease, bovine, further study of filtrable agent from (van den Ende & Turner) 225

Macromolecules, bacterial: isolation of deoxyribonucleic acid from *H. pertussis* (Overend *et al.*) xvi
Marcescin, antibiotic from *Serratia marcescens* (Fuller & Horton) 417
Media, partially defined, for *H. pertussis* (Ungar *et al.*) 345
Microcyst stage, morphology of, in differentiation of genera of Bacteriaceae (Bisset) 413
Microscopy, fluorescence, of tubercle bacilli (Matthaei) 393
Morphological changes of *V. cholerae* following cultivation in penicillin (P. Bruce White) 36
Morphological changes in *P. vulgaris*, *V. cholerae* etc., caused by penicillin (Fleming *et al.*) 257
Morphology of microcyst stage, in differentiating genera of Bacteriaceae (Bisset) 413
Motility of *P. vulgaris* on penicillin agar (Fleming) 457
Mutants, phage-resistant, of *Rhizobium trifolii* (Kleczkowska) 298
Mutation-rate of flagellar antigenic phase in *Salmonella typhi-murium*, measurement of (Stocker) vii
Mycobacterium phlei, differential resistance to licheniformin and subtilin (Hart & Moss) 244
Mycobacterium tuberculosis, fluorescence microscopy of (Matthaei) 393
Mycobacterium tuberculosis, resistance to infection of mice fed different diets (Howie) xi

New species, *Acetobacter acidum-mucosum* (Tosic & Walker) 192
New species, Actinomycetales, genus *Jensenia*, *J. canicruria* (Bisset & Moore) 280
New species, *Bacillus pantothenticus* (Proom & Knight) 539
New species, *Jensenia canicruria* (Bisset & Moore) 280
New species, *Lineola longa* and *L. articulata* (Pringsheim) 198
New species of aquatic nitrogen-fixing bacterium (Gray & Smith) 281
Ninhydrin-positive substances, characteristic extra-cellular, identified (Woiwod & Proom) 501
Ninhydrin-positive substances produced by bacteria, group and species specificity (Woiwod & Proom) v
Nisin, assay of (Hirsch) 70
Nitrogen-fixing bacterium, a new aquatic (Gray & Smith) 281
Nitrosomonas europaea, isolation in pure culture (Meiklejohn) 185
Nitrosomonas europaea, pure culture isolation of (Meiklejohn) vi
Nucleic acid, spectrophotometric estimation of, in bacterial suspensions (Mitchell) 399
Nutrient agars, peroxide-like inhibitor in (Proom *et al.*) 270
Nutrition of *H. pertussis* (Ungar *et al.*) 345
Nutrition of mesophilic species of genus *Bacillus* (Knight & Proom) 508

Obituary notice—S. Orla-Jensen (Olsen) 107
Orla-Jensen, S., Obituary notice (Olsen) 107
Osmophilic yeasts from concentrated orange juice (Ingram) ix

Pantothenic acid, characteristic nutrient for *B. pantothenticus* (n.sp.) (Proom & Knight) 539
Paracrinkle virus of potato, mechanical transmission of (Bawden *et al.*) 210; viii
Penicillin agar, motility of *P. vulgaris* on (Fleming) 457
Penicillin, effect of, on morphology and motility of *Proteus vulgaris* (Fleming *et al.*) 257
Penicillin, morphological changes in *V. cholerae*, caused by (P. Bruce White) 36
Peroxide-like inhibitor in nutrient agar (Proom *et al.*) 270
Phage-resistant mutants of *Rhizobium trifolii* (Kleczkowska) 298
Phage-typing of *Staph. aureus*, non-specific lysis observed in (Rippon) viii
Phenol, benzoic acid and related compounds, bacteriostasis of *Bact. aerogenes* by (Barbour & Vincent) 110
Pleuropneumonia group, biological properties and identification (Edward) 311
Pleuropneumonia-like organisms, in bovine genital tract (Edward) 4

Polypeptides, characteristic, in culture filtrates of *P. vulgaris* and *Cl. tetani* (Woiwod & Proom) 501
Potato paracrinkle virus, mechanical transmission and properties of (Bawden *et al.*) 210; viii
Potato virus *X*, aggregation of, studied with electron-microscope (Kleczkowski & Nixon) 220
Proteus vulgaris, characteristic polypeptides produced by (Woiwod & Proom) 501
Proteus vulgaris, effects of cobalt on growth and metabolism of (Schade & Levy) xvii
Proteus vulgaris, motility of, on penicillin agar (Fleming *et al.*) 257; (Fleming) 457
Polysaccharide synthesis from sucrose, liberation of reducing sugars during (Forsyth & Webley) 87
Purple bacteria, growth in defined media (Hutner) 286

Red-leg in tree-frogs caused by *Bact. alkaligenes* (Miles, E. M.) 434
Reducing sugars liberated during polysaccharide synthesis (Forsyth & Webley) 87
Rhizobium trifolii, phage-resistant mutants of (Kleczkowska) 298
Rumen bacteria, decarboxylation of succinic acid by washed suspensions of (Elsden & Sypesteyn) xi
Rumen bacteria, reduction of nitrate by (Lewis) xi

Saccharomyces cerevisiae, some aspects of cell division in (Barton) 84
Salmonella enteritis, resistance to infection of mice fed different diets (Howie) xii
Salmonella typhi-murium, flagellar antigenic phase, mutation-rate of (Stocker) vii
Sampling device, aseptic, for cultures (Heatley) 410
Serology of a yellow-pigmented Streptococcus (Hannay) 294
Serology of *Bacillus polymyxa* (Davies) xiv
Serratia marcescens, antibiotic marcescin from (Fuller & Horton) 417
Sexuality, queried, in *Fusiformis necrophorus* and *Streptobacillus moniliformis* (Klieneberger-Nobel) vi
Shigella dysenteriae, inhibition by peroxide in nutrient agars (Proom *et al.*) 270
Silica gel, improved method of preparation for culture media (Taylor) 235
Soil micro-flora, 'balance-sheet' of, tentative method for obtaining (Chesters & Apinis) vi
Spectrophotometric estimation of nucleic acid (Mitchell) 399
Spore-germination in *Bacillus*, chemical factors in (Hills) 38
Sporulation of *Cl. tetani* (Bisset) 1
Stalked forms of viruses (Robinow) 242
Staphylococcus aureus, a streptomycin-dependent strain (Foley & Shwachman) 141
Staphyloccocus aureus, assimilation of glutamic acid derivatives by (Gale) v
Staphylococcus aureus, non-specific lysis observed in phage-typing of (Rippon) viii
Staphylococcus aureus, treated with cetyltrimethylammonium bromide, release of cell constituents from (Salton & Alexander) ii
Starch-degradation by Group A streptococci (Crowley) 156
Starch-like polysaccharide formed by *Acetobacter acidum-mucosum*, n.sp. (Tosic & Walker) 192
Streptobacillus moniliformis, queried primitive form of sexuality in (Klieneberger-Nobel) vi
Streptococci in subacute endocarditis, classification of (Porterfield) 92
Streptococcus, a yellow-pigmented, serology of (Hannay) 294
Streptococcus, Group A, starch-degradation by (Crowley) 156
Streptomyces griseus, trace-element nutrition of (Chesters & Rolinson) i
Streptomycin-dependent strain of *Staph. aureus* (Foley & Shwachman) 141
Subtilin and licheniformin distinguished (Hart & Moss) 244
Subtilin, two opposing effects on bacteria (Asher) xvi

Technique for colony counts (Stirling *et al.*) 339
Tetrathionase; differential effect of temperature on growth and adaptation (Knox) 388
Tetrathionase; factors affecting rate of formation of (Jebb *et al.*) 374
Tobacco necrosis virus, factors affecting activation of preparations of (Bawden & Pirie) 464
Tobacco necrosis virus, infectivity of, effects of freezing and citrate on (Bawden & Pirie) 482
Toxicity of *Cl. bifermentans* and *Cl. welchii*, relation of lecithinases of (Miles & Miles) 22
Trace-element nutrition of *Streptomyces griseus* (Chesters & Rolinson) i
Tubercle bacilli, fluorescence microscopy of (Matthaei) 393

Urease in *Corynebacterium* spp. (Lovell & Harvey) 493

Viability of dried bacterial cultures (Rhodes) 450
Vibrio cholerae, effect of penicillin on morphology and motility of (Fleming *et al.*) 257
Vibrio cholerae, globular forms of (P. Bruce White) 36
Virus, fowl-plague, cytological studies on tissue cultures of (Challice & Flewett) xiv
Virus-infected eggs, amino-acid utilization in (van den Ende) 277
Virus of Lumpy Skin Disease of bovines, further study (van den Ende & Turner) 225

Virus, potato paracrinkle, mechanical transmission and some properties of (Bawden *et al.*) 210
*Virus, potato paracrinkle, mechanical transmission of (Bawden *et al.*) viii
Virus, tobacco necrosis, factors affecting activation of preparations of (Bawden & Pirie) 464
Virus, tobacco necrosis, infectivity of, effects of freezing in leaf and citrate *in vitro* (Bawden & Pirie) 482
Virus *X* of potato, aggregation of, studied with electron microscope (Kleczkowski & Nixon) 220
Viruses, plant, relationship between concentration of and numbers of local lesions (Kleczkowski) 53
Viruses, stalked forms of (Robinow) 242

Yeast, a strain requiring *p*-aminobenzoic acid (Cutts & Rainbow) 150
Yeast fermentation of glucose, effect of growth-factor deficiencies on (Hopkins & Pennington) 171
Yeast, formation of ethyl acetate by *H. anomala* (Peel) iv
Yeasts, osmophilic, from concentrated orange juice (Ingram) ix